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A Novel Vaccine with β_2-Microglobulin Linked to a Viral Epitope Stimulates a CTL Response and Provides Immunity to the Virus

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A Novel Vaccine with β_2-Microglobulin Linked to a Viral Epitope Stimulates a CTL Response and Provides Immunity to the Virus

by

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Dedication

This work is dedicated in loving memory to two wonderful women, Nora Agnes Piper and Ada Lee Whitock. Thank you grandmothers for showing me love throughout your lives.

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A Novel Vaccine with β_2-Microglobulin Linked to a Viral Epitope Stimulates a CTL Response and Provides Immunity to the Virus

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Designing a vaccine that can elicit a strong cytotoxic T-lymphocytes (CTL) response will be an important component of vaccines against many viruses, but in particular against HIV. CTL stimulation occurs through the recognition of epitopes presented in the context of the class-I major histocompatibility complex (MHC-I). As a way to achieve this, the physical coupling of a viral epitope to the N-terminus of β_2-microglobulin (β_2m) via a flexible linker has been explored. With the stimulation of CTLs against the Sendai virus as a model, both the effect of linker size on the vaccine and the ability of the β_2m-viral epitope fusion vaccine to trigger a primary immune response, as either a recombinant protein expressed in *E. coli* or as part of a mammalian expression vector are presented in this work. Here we show that as a DNA vaccine, β_2m-linked to the immunodominant Sendai $NP_{(324-332)}$ epitope, administered intramuscularly (IM) with or without a plasmid encoding for the cytokine IL-12, can cause the

proliferation of viral specific splenocytes. However, when the vaccine was administered IM or subcutaneously as recombinant protein, no response was observed. We also show that a construct with a 21 amino acid (AA) linker stimulates more viral specific splenocytes than constructs with either a 10 AA or a 15 AA linker. Further, DNA vaccine dosages of 100 μg and 200 μg generate more viral specific splenocytes than dosages of 400 μg or 800 μg. We suggest that the reason for this might be high-localized concentrations of β_2m. Finally, we show that in response to a sub-lethal challenge with the Sendai virus, mice primed with both the β_2m-Sendai epitope DNA vaccine and the IL-12 plasmid can trigger the early arrival of viral specific $CD8^+$ memory cells in the lungs.

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INTRODUCTION

Introduction to HIV

In 1981 M. S. Gottlieb et al., published in the New England Journal of Medicine the discovery of an immunodeficiency in highly sexually active gay men (Gottlieb, Schroff et al. 1981). This immune disorder would eventually be classified as the acquired immune deficiency syndrome, more commonly called AIDS. At the time the immune disorder was only seen in gay men and therefore it picked up a stigma as being a homosexual male disease. In 1983, the cause of AIDS was identified as the newly discovered lymphadenopathy-associated virus (LAV), which was later renamed the human immunodeficiency virus (HIV). Robert Gallo at the US National Institute of Health and Luc Montagnier at the Pasteur Institute along with their respective lab colleagues share this scientific feat (Prusiner 2002). In western Africa in 1986 a virus similar to HIV was isolated that was less pathogenic and immunologically distinct from HIV (Clavel, Guetard et al. 1986). The new virus was termed HIV-2 and the original HIV virus was given the new notation of HIV-1.

CURRENT STATE OF HIV IN THE WORLD

It has been shown that HIV can be found in blood, semen, vaginal fluids and breast milk, and can be transferred through contact with these fluids. HIV infection is indiscriminant of race, gender, or sexual orientation and generally AIDS is no longer seen as just a homosexual disease. According to the World Health Organization (WHO), since the discovery that HIV caused AIDS, more than 60 million people have been identified as being infected with the virus and the most common mode of transmission is heterosexual

exposure (Table 1.1) (WHO 2001). AIDS has become the fourth biggest killer in the world and the number one reason for death in sub-Saharan Africa. Here in the United States, AIDS has become the second leading killer of people between the ages of 25-44 years old. Worldwide over 20 million people have died of AIDS related diseases and with no vaccine or cure currently available the number of deaths is only expected to increase because of the other 40 million people currently living with the virus (WHO 2001). Furthermore, based on 2001 numbers this pandemic increased at a rate of 5 million people newly infected each year, which equates to approximately 14,000 HIV infections daily (Figure 1.1) (WHO 2001).

Region	Epidemic Started	People living with HIV	% Women	Main mode of transmission
Sub-Saharan Africa	Late '70s Early '80s	28.1 million	55 %	Hetero
South & South-East Asia	Late '80s	6.1 million	35%	Hetero, IDU
Eastern Europe & Central Asia	Early '90s	1 million	20%	IDU
North America	Late '70s Early '80s	940,000	20%	MSM, IDU, Hetero
Total		40 million	48%	

Table 1.1 Total number of people living with HIV in 2001.

The main mode of transmission whether it is via heterosexual contact (Hetero), intravenous drug use (IDU), or male sexual contact with males (MSM) is provided.

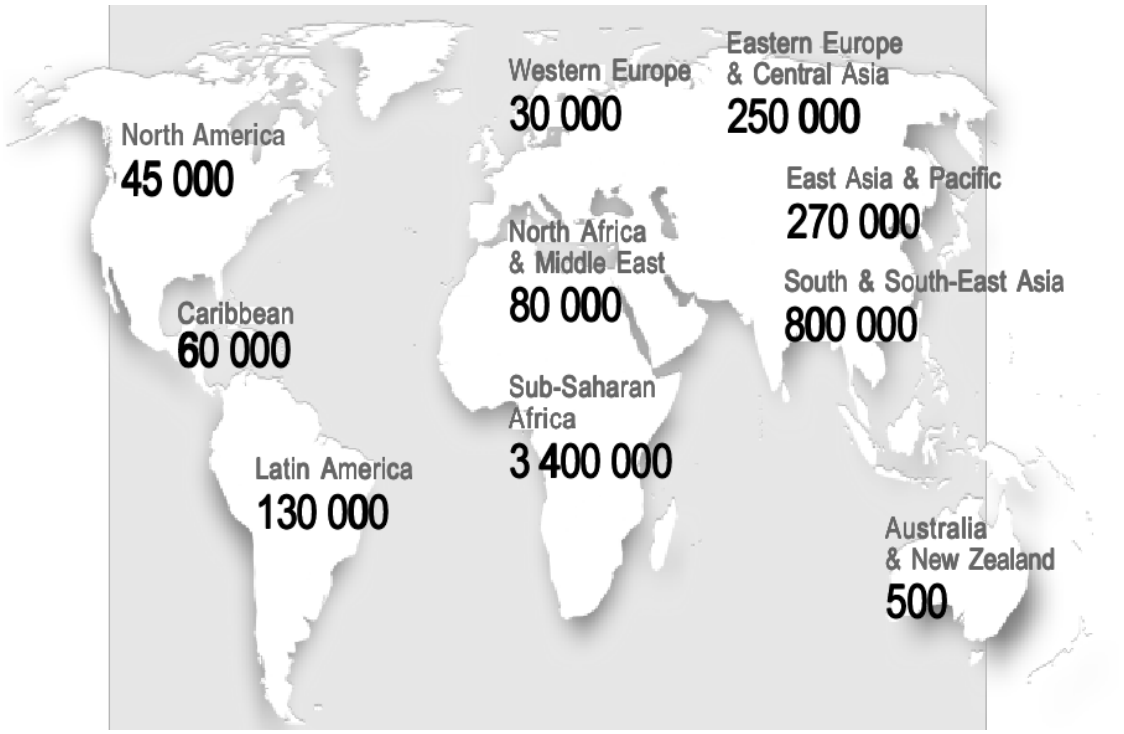


Figure 1.1 Estimated number of people newly infected with HIV during 2001. (WHO 2001)

The majority of those infected with HIV lived in developing areas such as sub-Saharan Africa and South-East Asia. The average life expectancy in sub-Saharan Africa has plunged by 15 years, from 62 years if AIDS was not a factor to 47 years with AIDS as a factor (WHO 2001). In addition to the loss of lives associated with HIV, the gross national product (GNP) for countries in this region has dropped by 0.5-1.2% and it is believed that by 2020 the GNP of the hardest hit countries (Botswana, Malawi, Mozambique, and Swaziland) may drop by more than 20% (WHO 2001). The influence of HIV on the GNP results from both the death of young adult wage earners and the financial strain of treating adults who have become infected and are eventually unable to work. HIV deaths have also played a toll in the education of young children. In 1999 it was estimated that 860,000 children lost teachers due to an AIDS related death (WHO 2001). In addition, the education of young women is expected to decrease further as more females are asked to provide care for HIV infected relatives.

To this point HIV infection in the world's most populated area of East Asia remains only at a very low prevalence rate. However, since HIV was not introduced to this area until the late 1980's and the area has yet to adopt an aggressive prevention program, countries like China and India sit primed for a major HIV pandemic. According to an update on the HIV epidemic in China published by WHO in June 2002, China lacks sufficient political commitment, has few effective policies, insufficient financial resources, and is deficient of healthcare personnel that possess the necessary medical training and education to prevent a major outbreak of HIV. Furthermore, HIV awareness in the general public remains low and a commitment to prevent transmission via sexual encounters or intravenous drug use appears small. China's Ministry of Health estimates that the number of HIV infections will exceeded 10 million by 2010 (China 2002).

How the HIV pandemic got started is not well known. The first reported case of HIV was found in a plasma sample of a Bantu man from Leopoldville, Belgian Congo (current day Kinshasa, Democratic Republic of Congo) who died in 1959 (Nahmias, Weiss et al. 1986). It is believed that he contracted the disease at some point in the late 1940's. Analysis of the amplified viral sequences places this sample of HIV near the ancestral node for HIV-1 subtypes B, D, and F (Zhu, Korber et al. 1998). This suggests that the beginning of the HIV pandemic may have evolved from the introduction of a single infection in the African population sometime shortly before 1959 (Zhu, Korber et al. 1998).

HIV INFECTION

As mentioned earlier, sexual contact, or more specifically heterosexual contact, has become the leading mode of transmission of HIV. When HIV enters at a mucosal site, such as the vagina or anal tract, the virus is believed to first infect or associate with Langerhans' cells (Lc). Lc cells are derived from macrophages and are located intertwined with the epithelial cells at the mucosal barriers. Lc cells express low levels of $CD4^+$ and the HIV co-receptor CCR5 (Dittmar, Simmons et al. 1997) and therefore can be infected by HIV. Lc cells also express a surface molecule known as DC-SIGN, which has a high affinity for the HIV surface protein, SU (also known as gp120) (Geijtenbeek, Kwon et al. 2000; Geijtenbeek, van Vliet et al. 2001). It is believed that instead of infecting the Lc, HIV bound to DC-SIGN is transported across the mucosal membrane. Once across the membrane the virus is released from DC-SIGN and infects nearby $CD4^+$/CCR5 T-cells. The infected T-cells, or Lc cells with HIV still attached, migrate to lymph nodes where the virus is given a chance to take hold (Figure 1.2). After about 2-6

weeks a burst of virus levels in the blood can be seen. This burst is known as the primary HIV infection, (PHI). The course of an HIV infection can be divided into two distinct phases the PHI and the secondary HIV infection (SHI) (Figure 1.3).

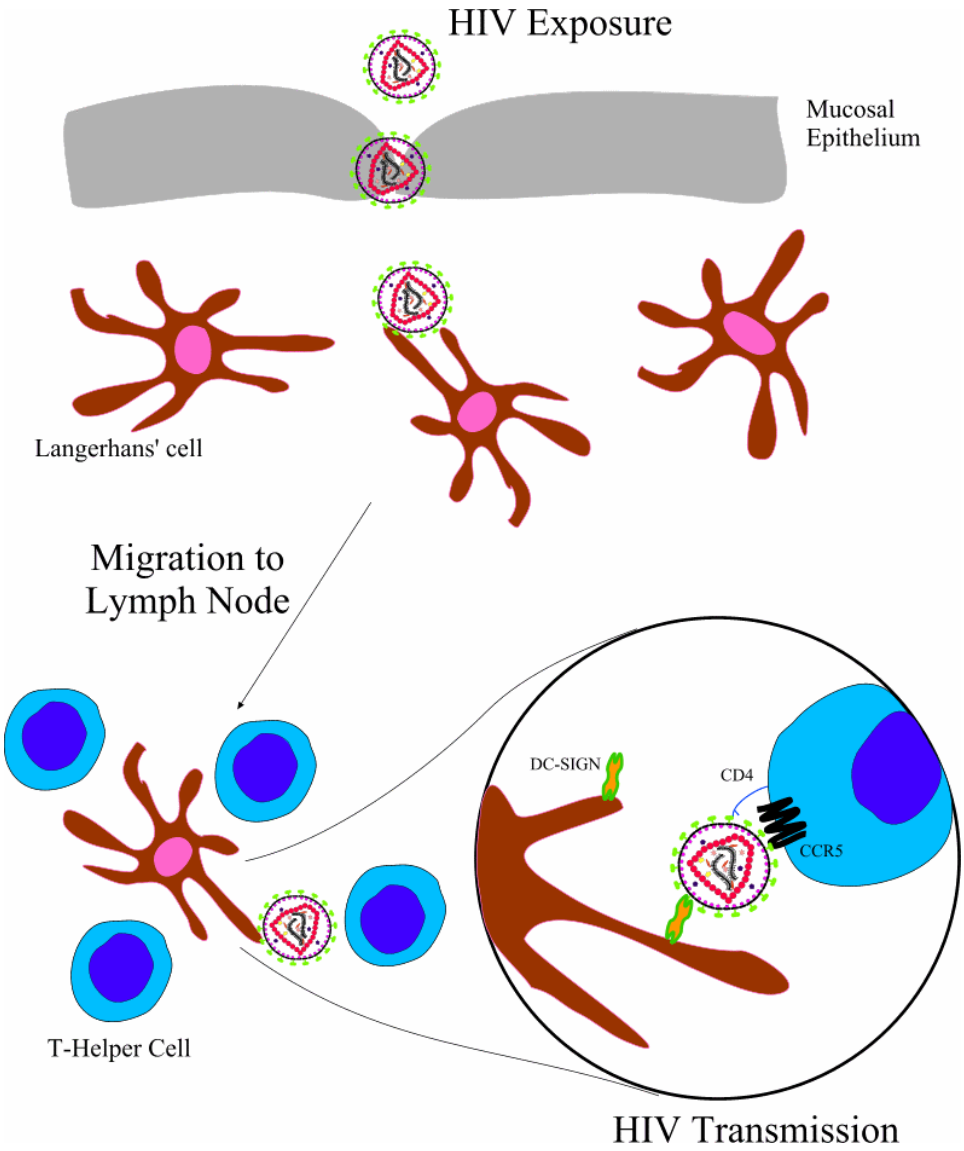


Figure 1.2 HIV Transmission via DC-SIGN on Langerhans' cells.

Modified from Geijtenbeek et al., 2001, figure 2 (Geijtenbeek, van Vliet et al. 2001).

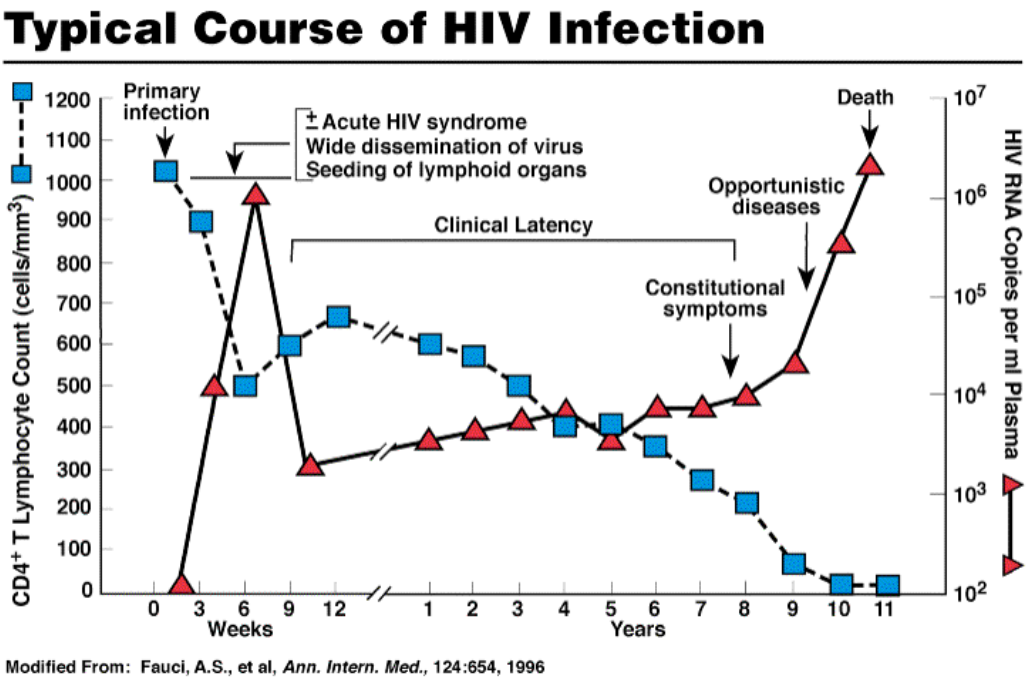


Figure 1.3 Course of an HIV Infection taken from McMichael, 2001 (McMichael and Rowland-Jones 2001).

The PHI is associated with the infections of $CD4^+$/CCR5 cells. PHI is characterized by an initial spike in the viral loads peaking at about 10^9 copies of HIV RNA per milliliter of blood. Following the initial increase of HIV RNA, there is a steady decrease, which coincides with an increase in HIV specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) (McMichael and Rowland-Jones 2001). During the first few weeks of infection there is a rapid decrease in the overall number of $CD4^+$ lymphocytes, from 800-1200 cells/µl seen in uninfected individuals to as low as 200 cells/µl during PHI, but generally it is not that severe (Weber 2001). The loss of $CD4^+$ cells is most likely due to a combination of direct virus lysis and $CD8^+$ CTL lysis of the infected $CD4^+$

cell. Patients in the PHI phase of infection may experience the development of rashes or the reoccurrence of previous infections but often they are asymptomatic.

After about several months the $CD4^+$ levels increase and levels out at a point below the pre-infection level. Viral RNA levels will remain low but a gradual linear decrease in $CD4^+$ cell counts will occur. The production of HIV virus particles has been estimated between 10^7 and 10^8 virions per day (Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995). While the average life expectancy for an HIV infected $CD4^+$ cell during this time is 24-48 hours as compared to an uninfected $CD4^+$ cell expected lifetime of 100 days (Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995; Perelson, Neumann et al. 1996). The infection will remain this way for 1-10 years without treatment depending on the host's genetics, characterizing the stage known as SHI.

Once the $CD4^+$ cell count has dropped below 200 cells/μl, the individual is classified as having AIDS. A switch in the HIV co-receptor from CCR5 to CXCR4 characterizes this last stage of HIV infection. The occurrence of this switch can be traced to the V3 loop of the SU (gp120) protein, where a 2 amino acid substitution directs the change. The CCR5 co-receptor is expressed in activated T-cells but is generally not expressed in the resting T-cell population. The CXCR4, on the other hand, is expressed on both resting and active T-cells. (Weber 2001) This switch gives HIV a greater pool of T-cells to infect and as a result one begins to see a more rapid decrease in the number of $CD4^+$ cells. The most life threatening opportunistic infections generally occur in greater frequency in persons with $CD4^+$ cell counts at less than 100 cells/μl (Carr, Chuah et al. 2000). For a list of these opportunistic diseases visit the CDC website at www.cdc.org.

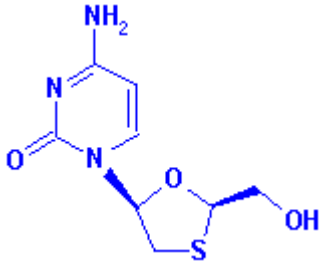
HAART

Persons infected with HIV experience a gradual deterioration of immune function over a period of 1-10 years if left untreated (Rutherford, Lifson et al. 1990; Cao and Walker 2000). If treated with the highly active antiretroviral therapy called HAART the patient can experience a higher quality of life and a longer survival time (Carr, Chuah et al. 2000; Van Vaerenbergh 2001; Fumaz, Tuldra et al. 2002; Gallant 2002). HAART was introduced in 1996 as a drug cocktail treatment consisting of two nucleoside analogues and a powerful protease inhibitor (Figure 1.4). Examples of these cocktails are zidovudine-lamivudine-indinavir (ZDV-3TC-IDV), stavudine-lamivudine-indinavir (d4T-3TC-IDV) or stavudine-didanosine-indinavir (d4T-ddI-IDV) (Carr, Chuah et al. 2000). Generally these drugs need to be taken twice a day at set time points. For effective reduction in viral load, as measured by viral RNA in the blood, recipients must have an overall adherence to the regime of greater than 95%, which corresponds to only missing one dose every 3 weeks (Paterson, Swindells et al. 2000; DeMasi, Graham et al. 2001). Along with stringent adherence requirements most HAART patients experience some adverse side-effects and as many as 70% experience potentially serious side effects (Harrington and Carpenter 2000).

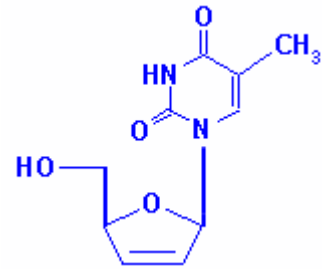
Economic arguments based on the cost effectiveness analysis of prevention verse treatment with HAART in developing countries with limited resources inevitably point towards efforts centered around prevention (Creese, Floyd et al. 2002; Marseille, Hofmann et al. 2002). Even though the cost of HAART per year per patient in developing countries is $350 compared to the US where the cost is $10,000 (Perez-Casas, Herranz et al. 2001; Boelaert, Van Damme et al. 2002), on a strictly economical argument money spent on the education of the population about the cause of AIDS and how to protect themselves from HIV infection still shows to be more effective. In the

Nucleoside Analogues Designed to Inhibit the HIV Reverse Transcriptase

Zidovudine or AZT



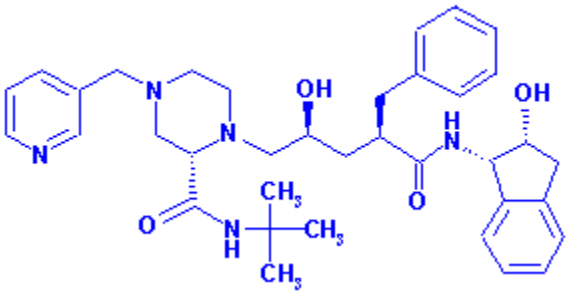
Lamivudine



Stavudine

Didanosine

HIV Protease Inhibitor



Indinavir

Figure 1.4 Chemical structure of drugs used in highly active antiviral therapy

2003 State of the Union Address, President G.W. Bush made history with the unprecedented announcement that the US would contribute 15 billion dollars over the next five years towards HIV efforts in Africa. If the sum of the money was ear marked for antiviral drugs, a very conservative estimate, performed by the author, based on the number of people living with HIV (28.1 million) (WHO 2001) multiplied by the cost of HAART for patients in developing countries ($350) gives a total cost of HAART treatment per year of 10 billion dollars per year. At 10 billion/year the US donation would be depleted in 1.5 years of HAART. The U.N. has made it known that the availability of HAART should increase in developing African countries because not all policy decisions are based solely on cost effective analysis (Boelaert, Van Damme et al. 2002). However, the cost of HAART certainly points strongly towards the need for an inexpensive vaccine.

HIV VACCINES

Vaccines in the past have functioned by reducing the viral load, during the acute phase of the infections, to a level at which the immune system is able to keep the infection clinically silent. It is unknown whether this strategy will work for an HIV vaccine because the virus infects the T-lymphocytes responsible for keeping the virus in check. This author has an optimistic point of view that believes sterilizing immunity, where the virus is completely eradicated from the host, will not be a necessity.

"The possibility for natural immunity to efficiently control virus infection makes it possible to believe that, if the mechanisms of survival are known, we will be able to generate a successful HIV vaccine" (Bojak, Deml et al. 2002). There is comfort in the knowledge that there are individuals who have been highly-exposed but persistently

seronegative (HEPS) (Devito, Hinkula et al. 2000; Dorrell, Hessell et al. 2000; Kaul, Rowland-Jones et al. 2001; Rowland-Jones, Pinheiro et al. 2001; Makedonas, Bruneau et al. 2002) and there are also infected individuals who have been classified as long term non-progressors (LTNPs) (Clerici and Shearer 1996; Candotti, Costagliola et al. 1999; Paroli, Propato et al. 2001). LTNPs are persons who maintain a stable $CD4^+$ cell count around 500 cells/μl and a viral load consistently below 500 HIV RNA copies/ml for 10 years or more. These people have demonstrated that the human immune system is able to keep the virus in check over a long period of time.

During the search for an effective vaccine we have learned many important contributors for successful control. A successful HIV vaccine must meet the requirements of any vaccine in that it must be safe, easily administered, generate a memory response and have long-lasting effects. The vaccine should be easily manufactured, stable for delivery, and economically feasible to produce in mass quantities. More specific requirements for an HIV vaccine are that it generates broadly cross-clade reactivity through both a robust neutralizing antibody response and a vigorous CTL response at both systemic and mucosal points of entry (McMichael, Mwau et al. 2002). The systemic immune response is needed to protect individuals from infection via blood transfusions or the sharing of needles by intravenous drug users. Although infection via blood transfusion has virtually been eliminated in this country it still remains a problem elsewhere in the world (Rosenthal 2002). The mucosal immune response is needed to protect individuals from sexually transmitted infection.

Early HIV vaccine designs attempted to trigger a neutralizing antibody response against HIV envelope proteins gp120 and its precursor gp160 (Arthur, Bess et al. 1989; Belshe, Graham et al. 1994; el-Amad, Murthy et al. 1995; Girard, Yue et al. 1996; Xie, Pestano et al. 1996). These vaccines showed promise because they provide protection

against laboratory strains of HIV, however they had trouble providing protection against primary HIV infection, (reviewed in (Moore 1995)). The reason for the failure of these vaccines was elucidated by Sullivan et al, 1995, when they showed that the antibodies generated against the monomeric form of the HIV envelope proteins could not recognize the trimeric form presented on the surface of HIV virion (Sullivan, Sun et al. 1995).

Recent evidence has pointed to the need to generate a cytotoxic T-lymphocyte response to control HIV and thus more current HIV vaccine designs have attempted to trigger this side of the immune response (reviewed in ((Letvin 1998)). CTLs are the cytotoxic arm of the cellular immune response. They can exert substantial pressure on HIV replication during both the primary and chronic stages of infections, which results in the development of viral escape mutants generated by the error prone HIV reverse transcriptase. It is postulated however, that if the CTL response is great enough, infected cells can be killed before they allow the virus to mutate (McMichael and Rowland-Jones 2001). This hypothesis is supported by the consistent presence of a cellular immune response specific to HIV in highly exposed persistently seronegative (HEPS) cohorts (Miyahira, Murata et al. 1995; Rowland-Jones, Dong et al. 1999; Kaul, Rowland-Jones et al. 2001; Rowland-Jones, Pinheiro et al. 2001; Li, Promadej et al. 2002). Also, HIV specific CTL responses have been detected in uninfected exposed health care workers and uninfected babies born to infected mothers (Pollack, Zhan et al. 1997; Buseyne, Burgard et al. 1998; Buseyne, Chaix et al. 1998; Riviere and Buseyne 1998; Wasik, Wierzbicki et al. 2000).

No HIV vaccine has successfully complete phase III studies. However there is one candidate that has finished phase III trials and two others who will start phase III trials later this year. VaxGen, a small pharmaceutical company based in Brisbane, CA, leaded the way with its recombinant HIV gp120 envelope protein, which completed

phase III trials in both the United States and Thailand, in February 2003. Unfortunately the vaccine failed to offer general protection to the population (McCarthy 2003). It has been shown that the rgp120 vaccine can stimulate an antibody response that reaches a maximal serum titer after three or four doses (Graham, Matthews et al. 1993). The rgp120 vaccine was unable to generate an infective class I major histocompatibility complex (MHC-I) CTL response, which will be required in order to provide protection against the virus. Therefore, it is believed that this vaccine will be most effective when combined with another approach that can induce the CTL response. With that in mine VaxGen combined with the US military, the Royal Thai government, Mahidol and Aventis has put forth another vaccine candidate that incorporates a Canarypox vector, which delivers the gag, protease, and env and is administered in conjunction with the rgp120 protein. This vaccine is able to generate the required broad CTL response along with antibodies against the glycoproteins and should start phase III trials in Thailand later this year. The third vaccine candidate, sponsored by a large contingent that includes the NIH, Aventis, Pasteur, and VaxGen, also utilizes Canarypox as the vector. This vaccine, in addition to the gag, protease, env, also codes for the pol and nef proteins, and like the other vaccine is administered in combinations with the rgp120 protein. This vaccine will also start phase III trials this year.

ANIMAL MODELS

Unfortunately, from a treatment standpoint, HIV-1 is a virus specific for humans and chimpanzees. Therefore, few options exist for experimental models that can help answer questions concerning the differences observed in the progression to AIDS in HIV-1 infected people. When chimpanzees are infected with HIV-1 isolated from

patients who have developed AIDS, the chimpanzee experiences an infection similar to the primary infection in humans (Arthur, Bess et al. 1989). However after several weeks the chimpanzee is generally asymptomatic, virus cannot be isolated from the blood, and the animal fails to progress to the clinical state of AIDS (Novembre, Saucier et al. 1997). The endangered species status of chimpanzees coupled with their failure to develop AIDS makes the chimpanzees a flawed choice for an animal model.

Once it became apparent that the chimpanzee model would not suffice, two options were explored: genetically created animals susceptible to HIV or the use of other animal viruses that are similar to HIV. Both pathways have been pursued. In the case of altered animals, scientists have created many mice models using transgenic mice and severe combined immunodeficient (SCID) mice. Transgenic mice are mice that have been altered so their genome contains one or all of the HIV genes. These types of mice have been used to examine select aspects of HIV infections. However the common problem with this model is an inability to mimic the entire HIV life cycle, especially the process involved during viral entry, reverse transcription, and integration (Freed and Martin 2001). Furthermore, transgenic mice show either very little or no productive infection. SCID mice are mice that are depleted of their own immune response and engrafted with human immune response components. These mice have also been useful for examining isolated aspects of HIV infection. Yet, like transgenic mice, this model is limited by an inability to present the entire HIV life cycle. They falter in modeling the types of tissues affected and in displaying the response generated by an intact immune system (Bieniasz and Cullen 2000).

Some recent work conducted in rats appears promising. It has been shown that rats engineered to express human CD4 and human CCR5 allow for cellular entry, replication and the production of infectious HIV virion (Keppler, Yonemoto et al. 2001).

Furthermore, these rats have shown the ability to develop AIDS-like complications (Reid, Sadowska et al. 2001). Because HIV is a human specific virus and the work in rats is relatively new the encouraging preliminary data should be taken with caution.

The second method of exploring HIV is to look at the effect other lenti retroviruses have on their respective host. Both the simian immunodeficiency virus (SIV) and the feline immunodeficiency virus (FIV) have been examined as potential models. There are six known SIV viruses that have been identified based on the host from which the virus was isolated, they are: SIV_{mac} from macaques, SIV_{smm} from sooty mangabey, SIV_{cpz} from chimpanzees, SIV_{mnd} from mandrill, SIV_{agm} from African green monkeys and SIV_{syk} from Syke's monkeys (Lewis and Johnson 1995). SIVmac in macaques is the only one of the six viruses that has similar characteristics to HIV infections. This model exhibits an initial high viral load, followed by a strong host immune response and a latent period of 1-3 years where CD4 counts steadily decrease, and an end of life due to the animal eventually succumbing to opportunistic infections (Lewis and Johnson 1995; Joag 2000). Like chimpanzees, macaques are an endangered species and are expensive to house, therefore only small scale studies are possible.

Although cats are relatively inexpensive and not on any endangered species list, there are some inherent problems with FIV. In the lab cats generally show the first signs of infection but rarely develop into an immuno-deficient state. Furthermore, it appears as though lymphocyte infection is independent of the $CD4^+$ molecule. The lack of animal models has been and unfortunately looks like it will continue to be a problem for HIV vaccine development.

The HIV Pathogen

This chapter is provided to give a brief explanation of the structure of the HIV virion and the function of each of the HIV viral proteins. It will also provide a general overview of the viral life cycle. For more details please refer to a recently published graduate level virology book such as Fundamental Virology, 4th edition edited by Knipe and Howley (Knipe and Howley 2001) or two recent reviews; one by Frankel and Young published in the Annual Review of Biochemistry (Frankel and Young 1998) and the other by Freed published in Virology (Freed 1998).

SUBTYPES

The human immunodeficiency virus (HIV) is a member of the lentivirus genus in the *Retroviridae* family. Phylogenetic analyses of HIV-1 isolates from various parts of the world have identified many different clades, or subtypes, which can be divided into three main groups: M (major), O (outlier) and N (non-M or O) (Figure 2.1). When the genome sequences from the M group are compared with sequences from the O group, they share less than 50% identity. Additionally the M group, which contains over 95% of HIV-1 sequences, includes 8 distinct clades, A, B, C, D, F, G, H and J. Genetic variations between clades may differ up to 30-40%, depending on the isolated genomes compared (Freed and Martin 2001). Not only do different isolates from around the globe vary from one another, different isolates from the same individual can vary in nucleotide sequence by as much as 10% (Freed and Martin 2001). The enormous variety of HIV-1 subtypes makes the task of developing a prophylactic vaccine more difficult.

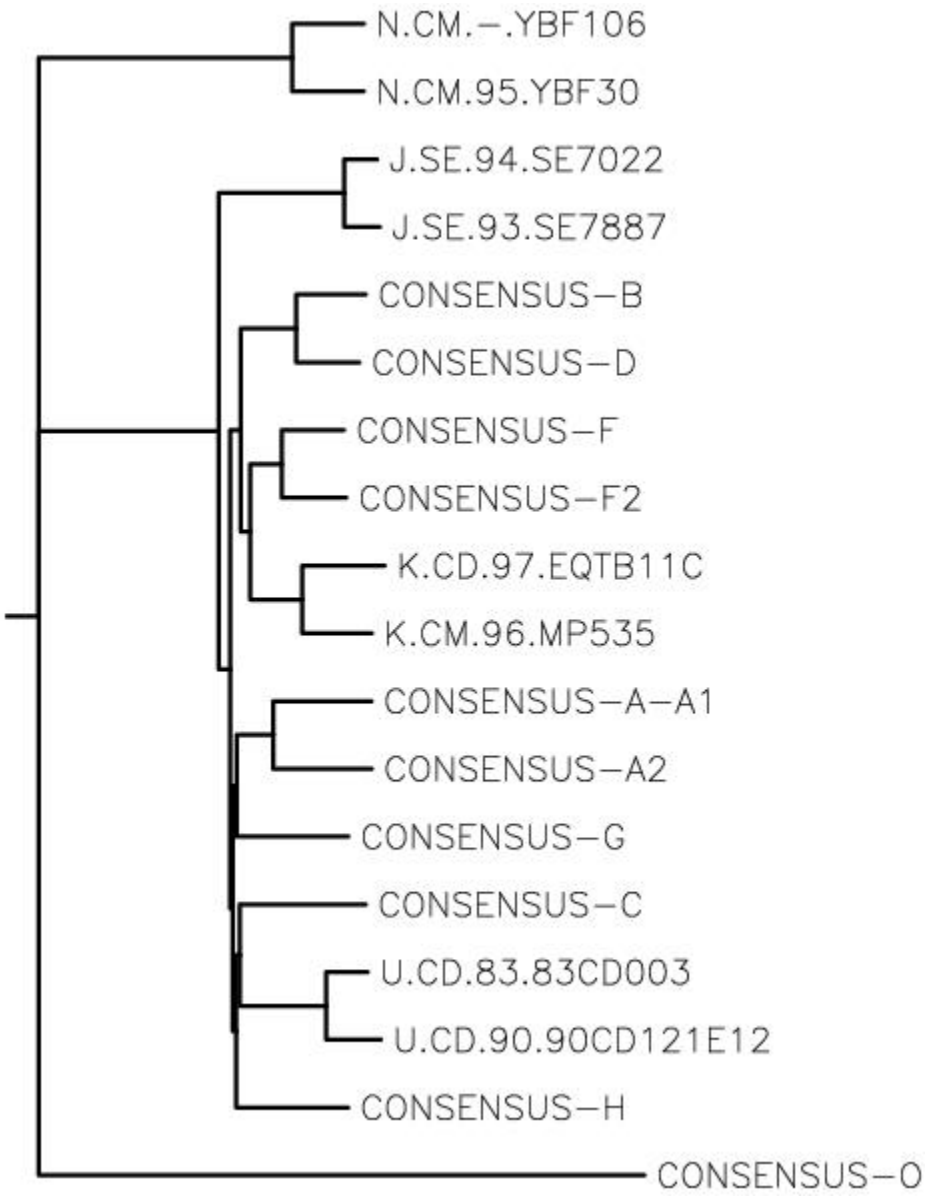


Figure 2.1 An HIV-1 Phylogenetic Tree for the HIV Pol DNA sequence.

This tree was created on 5/1/03 from files in the HIV Sequence Database, which can be found at http://hiv-web.lanl.gov/content/index.

HIV ORGANIZATION

HIV, like other retroviruses, has an enveloped virion with a diameter between 100-120 μm. A mature particle has a cone-shaped core that contains duplicate copies of the viral genome (Figure 2.2). The HIV genome is a single RNA strand that is approximately 9 kilo-bases in size and encodes 15 proteins. The genome encodes for 3 groups of structural proteins, 2 regulatory proteins, and 4 accessory proteins. The 3 groups of structural proteins, the group-specific antigen (Gag), the polymerase (Pol), and envelope (Env), are synthesized as three precursor poly-proteins. The precursor proteins Gag, Gag-Pro-Pol, and Env, are then post-translationally modified into the matrix protein (MA), the capsid protein (CA), the nucleocapsid protein (NC), the p6 protein, the protease (PR), the reverse transcriptase (RT), the integrase (IN), the surface protein (SU), and the transmembrane protein (TM). The two regulator proteins Tat and Rev are the first two proteins translated from spliced mRNA. The final four proteins, Vif, Vpr, Vpu, and Nef are also translated from spliced mRNA. These last four proteins are termed accessory proteins and were originally considered unnecessary because open reading frame (ORF) disruptions to these genes showed no effect on the virus's capacity to replicate and infect cells *in vitro*. Since *in vitro* experiments seldom have the ability to mimic all aspects of the *in vivo* environment, it was not surprising to find later that these proteins play vital roles *in vivo*.

In addition to the genes that code for viral proteins, the HIV genome also contains short terminal repeats. During reverse transcriptions (discussed later) these short terminal repeats increase in length and are then aptly termed long terminal repeats (LTR). The similar LTRs on the ends of the viral DNA are comprised of three functional regions known as: the unique 5′ region (U5), the unique 3′ (U3), and the repeat region (R). These LTRs play important roles throughout HIV's life cycle.

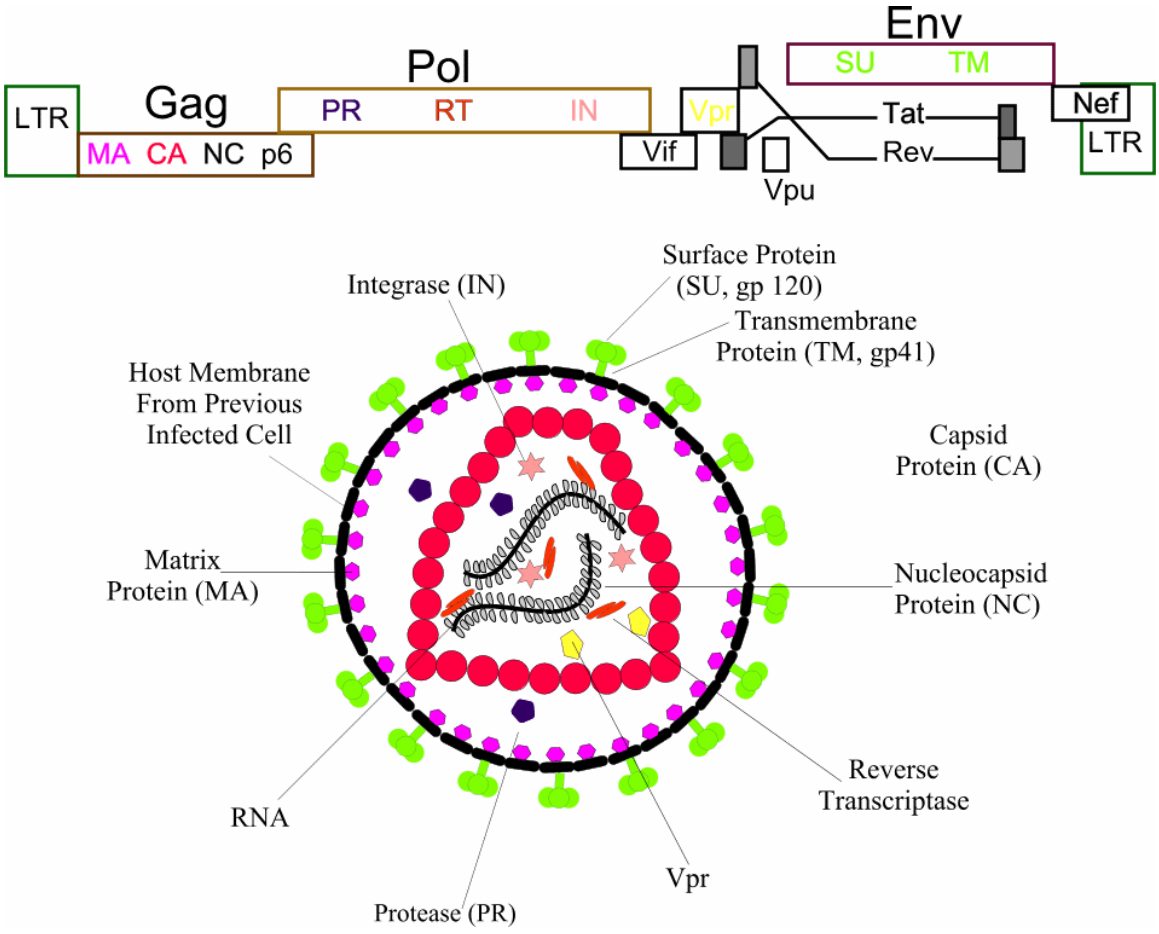


Figure 2.2 Schematic depiction of the HIV Genome and Virion,

Adapted from Figure 1 Frankel and Young, 1998, (Frankel and Young 1998)

TRANSCRIPTION AND REPLICATION

Starting from the inserted viral DNA, the life cycle of HIV begins with the initiation of transcription (Figure 2.3). The transcriptional promoters, enhancers, and TATA box necessary to bring RNA polymerase II to the start site are located in the U3 region of the LTR. Of particular interest are the two binding sites for the NF-κB transcription factor, which is turned on in active T-cells. With the help of the cellular

transcription factors the RNA polymerase engages at the HIV start site located at the interface between the U3 region and the R region of the 5' LTR. The polymerase then proceeds to transcribe the viral gene products.

There are three classes of viral RNA that leave the nucleus. The first class consists of full-length transcripts that serve both as the genome for progeny virus and the mRNA for the Gag and Gag-Pro-Pol precursors. The second is incompletely spliced RNA that provides the message for Vif, Vpr, Vpu and Env proteins. The final type is fully processed mRNA that codes for the Tat, Rev, and Nef proteins.

Only very low levels of viral transcription are seen until the viral gene product Tat is recruited back to the nucleus. The Tat protein of HIV is responsible for increasing production of viral RNA by over 100 fold when compared to the steady-state levels of viral RNA. In Tat deficient mutants transcription often does not proceed more than a few hundred nucleotides and viral progeny are not produced (Frankel and Young 1998). The 86 amino acid protein, Tat, consists of two distinct regions known as the activation domain, amino acids 1 through 48, and an arginine rich RNA binding region, amino acids 49-57. Tat's activation domain recruits the cellular cofactors cyclin T1 and CDK9 to RNA hairpin regions known as TAR (trans-activating response elements) that form at the start of viral transcription (Freed and Martin 2001). Tat's RNA binding domain allows Tat to bind to TAR. The recruited cofactors phosphorylate the C-terminal domain of RNA polymerase II (Freed and Martin 2001). The phosphorylated RNA polymerase II then proceeds to transcribe the remaining viral RNA.

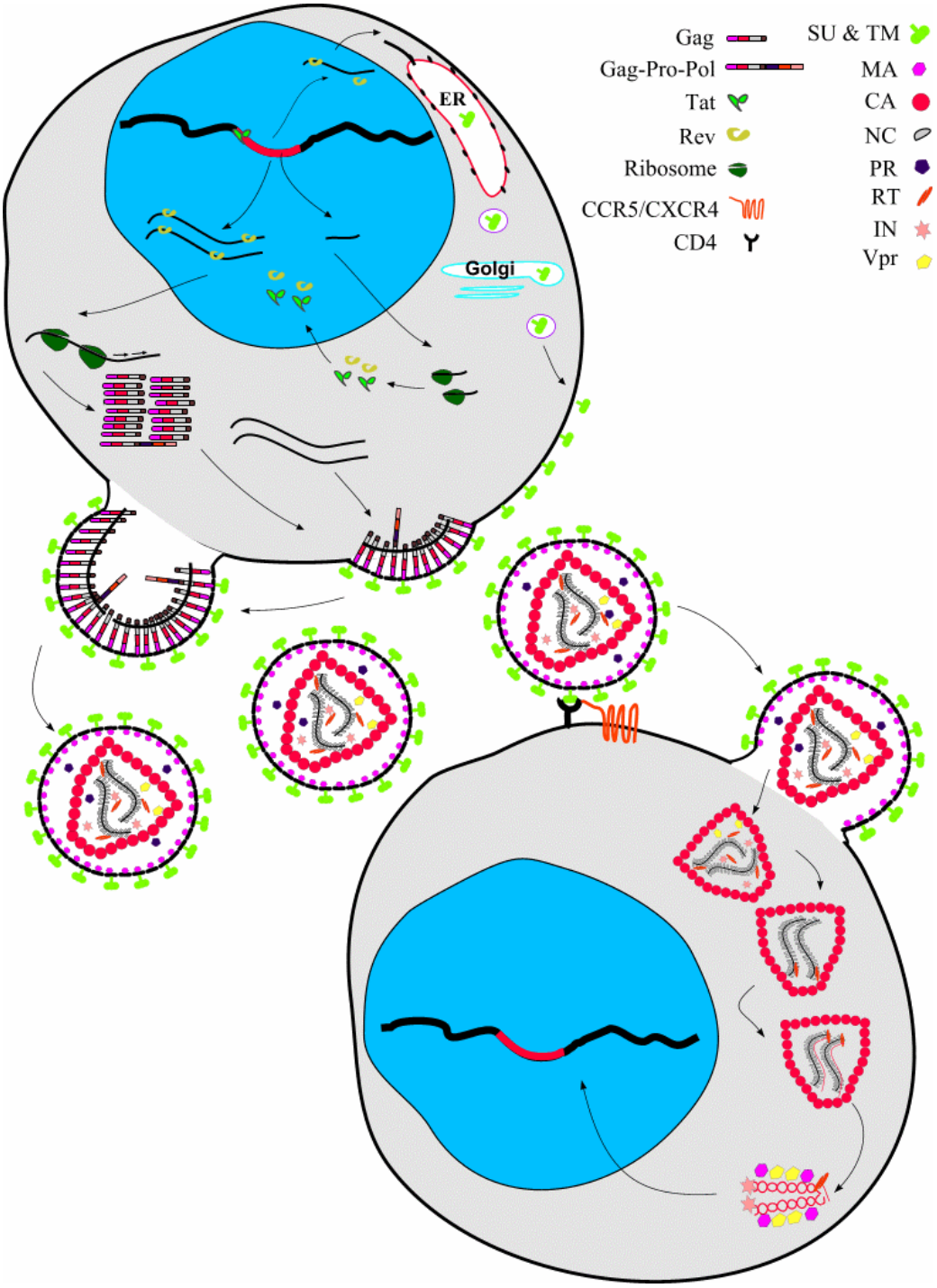


Figure 2.3 Schematic depiction of the HIV life cycle.

The cycle is more fully described in text.

HIV's 116 amino acid Rev protein, which stands for **r**egulator of **e**xpression of **v**iral protein, is responsible for transporting unspliced and incompletely spliced viral mRNA out of the nucleus (Vaishnav and Wong-Staal 1991). Generally before leaving the nucleus introns in mRNA are removed to prevent the translation of non-functional proteins. However a method must exist to get the unmodified RNA out of the nucleus since the HIV RNA genome as well as the partially spliced RNA must get into the cytoplasm. The Rev protein is responsible for accomplishing this task. Rev deletion mutants show no accumulation in the cytoplasm of the unspliced RNA or the partially spliced mRNA for Vif, Vpr, and Vpu/Env. (Freed and Martin 2001)

The Rev protein has two functional domains. The first domain has a binding site for a 60 nt portion of the Rev response element (RRE). RRE is a cis-acting target on all unspliced and partially spliced viral mRNA (Freed and Martin 2001). This domain of Rev also contains a nuclear localization signal that calls translated protein back to the nucleus. The second Rev domain contains a leucine-rich nuclear export signal (NES), which gives it the ability to re-cross the nuclear membrane (Frankel and Young 1998). Through a complicated sequence of events Rev binds to the RRE on viral RNA and transports the RNA through a nuclear pore across the nuclear membrane (For more details see Freed and Martin 2001).

TRANSLATION

After the RNAs have escaped the nucleus, the host cell's machinery translates the viral gene products and poly-protein precursors: Gag, Gag-Pro-Pol, and Env. The Gag precursor, which accounts for over 90% of the viral structural proteins (Ott 2002), has an approximate molecular weight of 55 kilo-daltons and therefore is also sometimes referred

to as $Pr55^{Gag}$. As mentioned earlier, the unspliced viral RNA serves as the mRNA for Gag. This unspliced viral RNA also acts as the mRNA for the 160 amino acid Gag-Pro-Pol precursor, which is also known as $Pr160^{Gag\text{-}Pro\text{-}Pol}$ or just Gag-Pol. The Gag-Pro-Pol precursor forms after a stop codon is ignored during the translation of Gag. Gag and Pro-Pol lie in different open reading frames (ORF) and about 10% of the time, at a highly-uridine rich region between the Gag and Pro-Pol genes, the translating ribosome slips back one nucleotide (Parkin, Chamorro et al. 1992). This frame shift allows the ribosome to continue translating through the stop codon.

The mRNA that codes the Env precursor (gp160) is partially spliced to remove the majority of the Gag and Pro-Pol genes before leaving the nucleus. The first part of the Env precursor translated is a signal peptide that directs the majority of the remaining protein to be translated directly into the ER. A hydrophobic region near the 3′ end anchors the protein in the ER membrane. The leader sequence is removed from the protein on the lumen side and the protein is then heavily glycosylated and folded. The folded Env protein is transported to the Golgi complex where it is cleaved into the SU, (gp120) and TM (gp41) proteins by a cellular protease. These proteins are then transported to the surface of the host cell.

ASSEMBLY AND BUDDING

While the Env proteins are being transported to the surface of the cell, a membrane signal tells the Gag and Gag-Pro-Pol to gather near the plasma membrane. The myristic acid moiety attached to the N-terminus of the MA along with another N-terminal domain of the MA protein, known as the M domain, act as the signal for both precursors (Freed 1998). The MA portion of these precursors is also believed to interact

with the cytoplasmic tail of the TM protein to ensure the incorporation of TM and SU into the budding virus.

To ensure that the RNA genome is also packaged before budding, HIV utilizes the NC region of the poly-proteins. The NC protein contains a short sequence consisting primarily of cystine residues and a histidine residue that form a motif similar to a zinc-finger. This motif allows the NC protein to bind to various RNA packaging regions on the viral genome. The primary packaging region is generally found in between the LTR and Gag. RNA is found in mature virions as a dimer, however whether it is packaged as a dimer or forms a dimer during the maturation process is still not clear. Also, at some point during the RNA packaging and viral assembly processes, host cell tRNAs, in particular $tRNA^{Lys3}$, are integrated into the virion. Interactions between the tRNA and the RT portion of the Gag-Pro-Pol precursor are believed to be important.

After the assembly of the necessary viral components at the plasma membrane, the soon-to-be progeny virus enters the last stage of budding. As of now this stage is still not well known. But what is known is that mutations to the highly conserved Pro-Thr-Ala-Pro motif of the p6 portion of the Gag poly-protein prevent the virion from releasing effectively. The p6 portion is also responsible for making sure that the accessory proteins Vpr and Vpx are incorporated into the virion before budding.

MATURATION AND THE GAG PROTEINS

After budding the HIV virion is still not considered a mature virus particle, because at this point if it infects a new cell it is unable to reverse transcribe the viral genome. But that will not remain the case once the PR region of the Gag-Pro-Pol precursors is able to cleave itself out. PR acts as a homodimer that forms either because

of conformational changes to the precursor or because of the high concentration of precursor proteins in the virion compared to the host cytoplasm. Once dimerized the PR protein is able to cleave itself. After cutting itself out it then makes several cleavages in both the Gag and Gag-Pro-Pol precursors. The processing of these poly-proteins causes a conformational change that triggers the formation of a cone-shaped core around the viral genome. At this point the virus particle is able to infect new cells and is thus considered mature.

PR cleaves the Gag precursor into the MA, CA, NC and p6 proteins. The major role of the MA protein, which is also known as the matrix protein or p17, during viral assembly has been discussed earlier. But there is evidence that suggests the MA protein also plays a post infection role. MA mutants demonstrated impaired RT activity that lead to a decrease in viral DNA synthesis (Kiernan, Ono et al. 1998). MA has also been found in low amounts in the viral preintegration complex that transports viral DNA into the nucleus.

The 231 amino acid CA proteins form HIV's cone-shaped core that holds the viral genome. CA has two functional domains: one near its C-terminus and the other at its N-terminus. The mutations to the C-terminal domain inhibit the ability of CA to form dimers, which lead to assembly defects, whereas mutations to the N-terminal domain drastically impair the ability of the virus to infect host cells. The C-terminal region of the CA protein, along with the NC protein, is believed to help the 1,200-1,800 Gag precursors properly pack into a spherical shape during assembly. The N-terminal domain is believed to play a role in HIV core formation and is known to recruit the cellular protein cyclophilin A into the nucleus. Cyclophilin A is believed to destabilize CA-CA interactions post infection allowing the uncoating of the viral genome (Gamble, Vajdos et al. 1996).

The role played in RNA packaging, Gag assembly and tRNA incorporation by the 72 amino acid NC protein, while part of the Gag precursor, was mentioned earlier. In the mature detached virion NC can be found coupled with the viral genome in the cone-shaped core. NC has been shown to act as a nucleic acid chaperone binding to RNA at a ratio of one molecule per seven nucleotides (Rein, Henderson et al. 1998). NC mutants demonstrate decreased infectivity, which is believed to be associated with a destabilization of newly reverse transcribed viral DNA, which in turn leads to degradation (Tanchou, Decimo et al. 1998).

Outside of what was mentioned about viral budding, not much more is known about the role of the Gag protein p6. Cleavage of the other precursor Gag-Pro-Pol gives the additional proteins PR, RT, and IN. The role of the RT and IN proteins will be described in context with HIV's life cycle. As mentioned earlier, the role of the PR protein is as a protease making the necessary cleavages to the precursor proteins to give a mature viral particle. The active sites for the PR dimer are known but what makes a good cleavage site for the protease seems to follow a fairly relaxed set of governances that are not well understood. What is known is that the PR binding site can hold approximately seven amino acids and that PR has the affinity for a cleavage site between CA and NC.

BINDING AND ENTRY

After precursor processing has occurred the progeny virion is ready to infect a new cell. The heavily glycosylated SU molecule (gp120) interacts non-covalently with the smaller transmembrane protein, TM (gp41), to form a heterodimer. This heterodimer is then believed to form a trimer, which then serves as the binding complex for HIV (Lu, Blacklow et al. 1995). The SU portion of this complex binds to HIV's primary receptor,

the surface molecule CD4. CD4 is expressed primarily on T-helper (T_H) cells but is also seen to a lesser extent on dendritic cells and macrophages. The binding of CD4 alone does not facilitate infection; HIV must still interact with a co-receptor from the transmembrane proteins of the chemokine receptor family (Berger, Murphy et al. 1999). The binding to CD4, however, does cause a conformational change in the SU protein that either exposes or forms the binding site for the co-receptor molecule (Burton 1997). As mentioned earlier the two major co-receptors for HIV are CCR5 during PHI and CXCR4 during the transition to AIDS.

Once the co-receptor has become bound, the SU molecule experiences another conformational change that activates the TM protein. The activated TM protein is believed to insert itself into the host cell thus facilitating closer virion-cell contact (Poignard, Saphire et al. 2001). At this point, in a process that is not understood, the virus is able to penetrate the host cell and insert its viral core.

REVERSE TRANSCRIPTION

After the insertion of the viral core into the cell, HIV begins the process of reverse transcribing its RNA into DNA. This complicated process has been well studied and can be broken down into six steps (Figure 2.4) (Gotte, Li et al. 1999). Reverse transcription takes place in a large complex similar to the viral core. Most of the reaction is mediated by the activity of RT, however, it has been shown that NC may play a role in the stabilization of viral DNA ends. After analysis of viral DNA from NC mutants it was determined that the DNA had been truncated (Goff 2001).

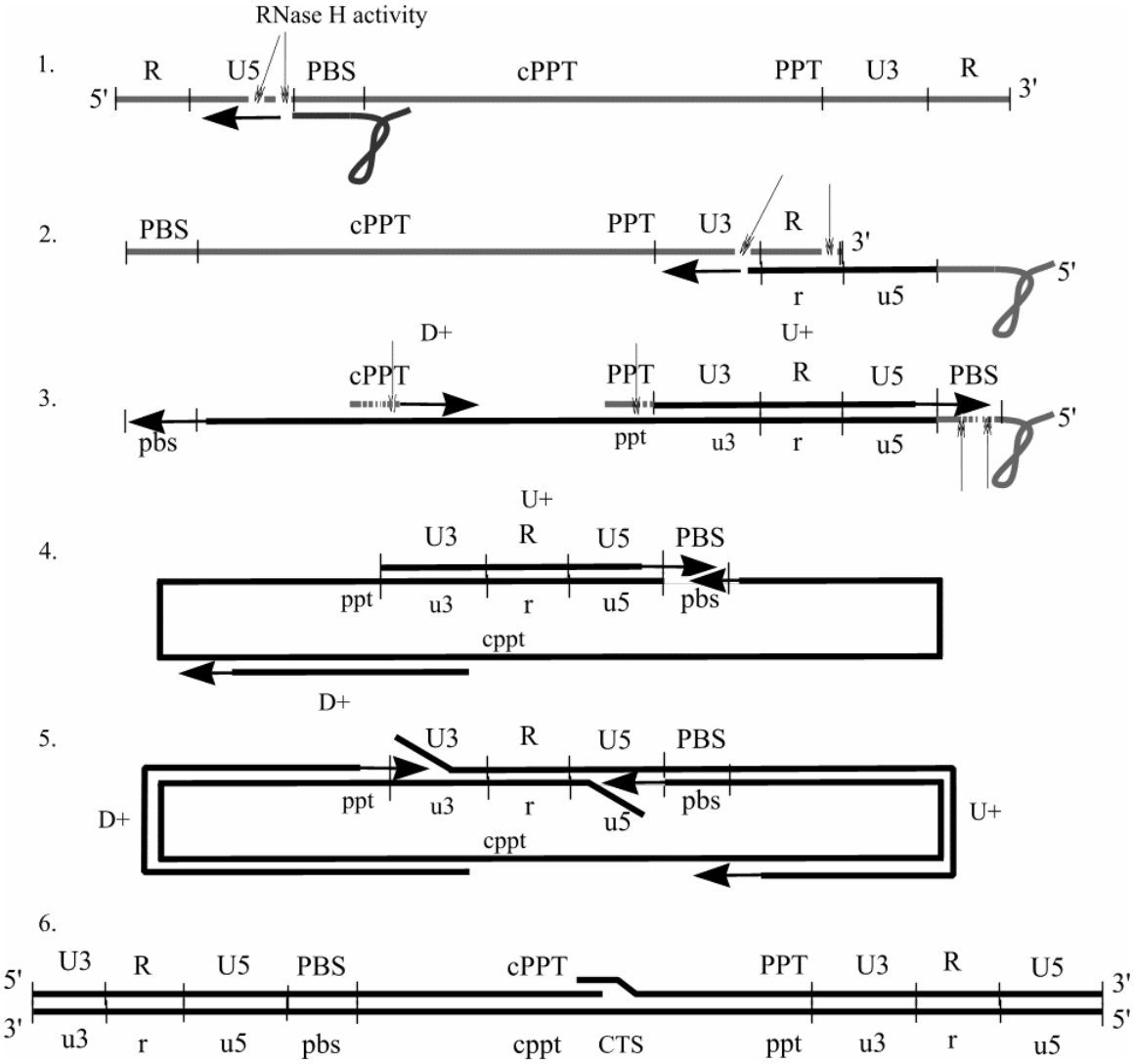


Figure 2.4 Schematic depiction of the Reverse Transcription of Viral RNA into Proviral DNA

Modified from (Gotte, Li et al. 1999) and described in text.

The active form of RT is a heterodimer consisting of a 66 kd and a 51 kd subunit to form a holoenzyme. The 66 kd subunit is considered the full length RT protein, while the 51 kd subunit comes about after a cleavage by PR of RT that cuts out the RNase H domain near its C-terminus. The holoenzyme possesses RNA polymerase activity, DNA polymerase activity, and RNase H activity.

During the first step, the tRNA from the previous host cell anneals to the primer binding site (PBS) on the viral RNA template. The PBS is located in the U5′ region of the RNA genome. This site is complimentary to 18 nucleotides at the 3′ end of the $tRNA^{Lys3}$. RT recognizes this RNA/tRNA complex and begins to extend the 3′ end of the tRNA using the viral RNA as the template to create a new RNA/DNA hybrid. Along with the ability to transcribe DNA from RNA, RT has RNase H activity, which allows it to degrade the RNA portion of the hybrid. This leaves only a DNA portion that is complementary to the viral U5′ and R regions of the genome.

The second step in the process of generating a full length DNA copy of the genome utilizes the fact that the HIV genome has similar R regions on both the 5′ and 3′ ends. The complimentary R region on the newly created piece of DNA binds the R region on the 3′ end of the viral genome. Since the viral genome is an RNA dimer it is not clear if the DNA binds the RNA strand it used earlier for a template or if it simply binds the other RNA genome.

During step three in the process RT begins transcribing a new full length piece of negative DNA. As before, but with one small exception, the RNase H activity of RT degrades the RNA from the newly formed RNA/DNA hybrid. The exception is that the full length RNA genome contains two purine rich regions that are resistant to RNA degradation. One region is located near the 3′ terminus and is termed the 3′ polypurine tract (3′PPT), while the other can be found in the center of the genome and is aptly

termed the central polypurine tract (cPPT). These regions will serve as the primer for the creation of a positive DNA strand.

Before the negative strand of DNA is complete the creation of a positive strand of DNA has begun at both the 3′PPT and cPPT. The DNA created from these starting points are termed upstream positive (U+) and downstream positive (D+), respectively. When RT on the U+ reaches the annealing point of the short negative DNA strand it continues transcribing to include the 18 nucleotides of the tRNA it used as a primer. The 19th nucleotide of the tRNA acts as a stop signal and is known as the plus-strand-strong-stop DNA. At this point RT uses its RNase H activity to chop up the tRNA as well as to go back and get rid of the previously un-digestible PPTs.

The fourth step in the process involves another strand transfer. This time the positive DNA strands U+ and D+ bind the negative full length DNA. RT then continues the transcription of the positive DNA strand using the negative strand as the template.

The fifth step of the process highlights another activity of RT. During the transcription of the positive strand RT is inevitably going to run into the point where the U+ or D+ bind. When this happens RT must have a way to disrupt the DNA/DNA interaction to allow the transcription to continue. RT continues transcribing the D+ strand until it reaches the PBS region. This continuation creates the 3′ LTR on the positive DNA.

The sixth step involves the continuation of the U+ strand transcription until it reaches a termination sequence in the center of the genome. This sequence is approximately 100 nucleotides downstream of the D+ 5′ binding site and is known as the central termination sequence (CTS). Because of CTS's location the positive DNA strand will have an overlap in the middle. The negative and positive DNA strands interact to give the proviral DNA.

INTEGRATION

After the conversion of RNA to proviral DNA, by the reverse transcriptase complex, the viral core proteins are shed to give the pre-integration complex (PIC) (Figure 2.5) (Sherman and Greene 2002). PIC is comprised of the proviral DNA, IN, MA, Vpr and RT. PIC gives the newly created proviral DNA the ability to cross the membrane of dividing and non-dividing cells. PIC utilizes host cell macromolecules (known as nuclear pore complexes (NPCs)) that span the nuclear membrane. The NPC is made up of 50-100 host cell proteins.

The IN protein of PIC contains a nuclear localization signal (NLS) somewhere between amino acids 152-184 (Bouyac-Bertoia, Dvorin et al. 2001). A yet-to-be determined host cell protein, which is most likely from the family of proteins known as importins, binds NLS and facilitates the passage of PIC through the nuclear membrane. Mutations in the NLS sequence of IN prevent the integration of proviral DNA in both dividing and non-dividing cells (Bouyac-Bertoia, Dvorin et al. 2001).

NPC allows passive transport of molecules with a radius of up to 9nm and active transport of molecules with a radius of up to 25 nm (reviewed in (Mattaj and Englmeier 1998)). The radius of PIC prior to import is estimated at 56 nm (Miller, Farnet et al. 1997). Therefore a method must exist that allows PIC to condense to a smaller size. The linear proviral DNA is 9.4 kilo nucleotides in length which when linear would equate to a length of ~ 3.3 μm. In order for the PIC to have an average radius of 56 nm, the proviral DNA is most likely coiled in some fashion. It is also known that IN binds the ends of the proviral DNA, which might provide protection from exonuclease activity (Miller, Farnet et al. 1997). After binding the INs then come together to form a multimer, causing the proviral DNA to fold (reviewed in (Cullen 2001)). The DNA overlap in the positive proviral DNA is also believed to provide additional flexibility to allow the PIC complex

to condense even further. It is known that HIV mutants that were engineered to be deficient of the overlap were not able to infect cells (reviewed in (Sherman and Greene 2002)).

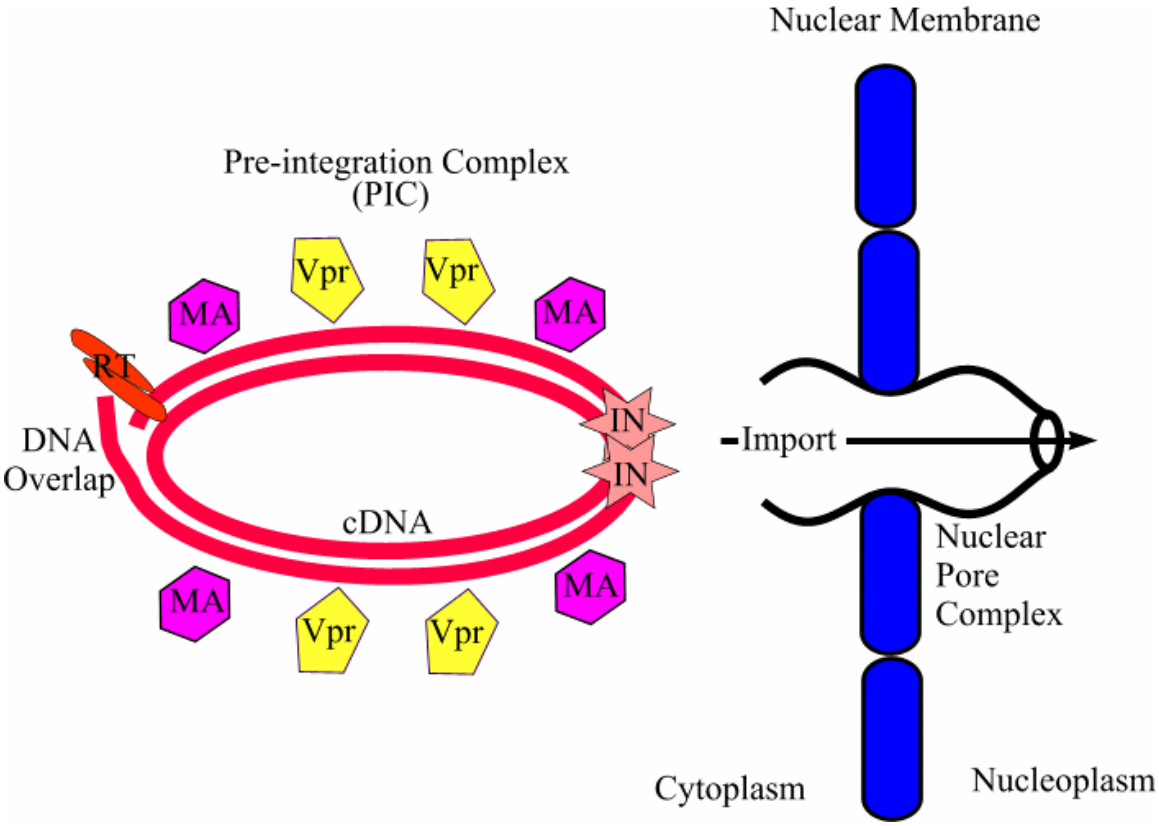


Figure 2.5 Schematic depiction of the Preintegration Complex with the Nuclear Pore Complex

Adapted from (Sherman and Greene 2002)

In addition to IN and the proviral DNA, the PIC also contains Vpr, MA, and RT. Vpr is believed to give HIV the ability to infect non-dividing macrophages. In culture Vpr deletion mutants show decreased viral replication in non-dividing macrophages but have little effect on replication in proliferating PBMC or T-cells (de Noronha, Sherman et al. 2001). The role of MA and RT in nuclear import is not understood.

After transversing the nuclear membrane, the proviral DNA must still be inserted into the host genome. This involves a 3-step process. During the first step the 32 kd IN removes 2-3 nucleotides from the 3′ ends of the proviral DNA to create overhangs. In the next step IN uses the 3′ OH to break the phosphodiester backbone of the host DNA and attach the 3′ end of the proviral DNA to the 5′ end of the cut in the host DNA. The location is sequence independent and results in the 3′ ends of the viral DNA attached to the host DNA and the 5′ end protruding. NC may also be involved in this step, because during *in vitro* integration assays without NC, the major product was only a single end of the proviral DNA attached to the host genome (Carteau, Gorelick et al. 1999). The final step of integration employs host cell machinery to remove the 5′ overhang and repair the DNA.

THE ACCESSORY PROTEINS

Vif stands for **v**irus **i**nfectivity **f**actor and this 192 amino acid protein is important for productive infection *in vivo* (Frankel and Young 1998; Potash, Bentsman et al. 1998; Blumenzweig, Baraz et al. 2002; Pomerantz 2002; Yang, Gao et al. 2003). Vif defective mutants *in vitro* have been shown to infect the cell lines 293T, COS, HeLa, SupT1, CEM-SS and Jurkat. These cells have been termed Vif-permissive. Other cells such as primary lymphocytes, some T-cell lines and monocyte-derived macrophages are not

infected *in vitro* by Vif defective mutants and are thus termed Vif-non-permissive (Freed and Martin 2001).

A much debated question is whether Vif is inhibiting a host cell factor found in non-permissive cells or is filling the role of a host cell factor absent in these cells. That debate has recently swung towards the argument that Vif is inhibiting a cell factor found in non-permissive cells. Sheehy et al. published in 2002 that a host cell factor from a non-permissive cell, when expressed in permissive cells, causes these cells to become non-permissive (Sheehy, Gaddis et al. 2002). They termed the host cell factor CEM15 after the cell line from which it was isolated.

Also, recently, an activity of Vif most likely unrelated to the inhibition of CEM15 has been reported. The N-terminal portion of Vif has been shown *in vitro* to inhibit the activity of PR, the HIV protease (Potash, Bentsman et al. 1998; Yang, Gao et al. 2003). The inhibition is believed to ensure that the PR refrains from cleaving itself out prematurely, thus allowing for proper assembly of the Gag and Gag-Pro-Pol precursor.

Vpr, for viral protein R, is a 96 amino acid protein believed to serve three functions. First, as mentioned earlier, Vpr is part of the PIC and is important during the infection of non-dividing macrophages. Second, it has been shown that T-cells infected with HIV-1 containing an intact Vpr gene product do not replicate and eventually die (reviewed in (Emerman 1996)). Vpr locks the T-cell in the G2 phase preventing it from entering mitosis. The highest rate of HIV expression is seen in the G2 phase, which might explain why Vpr locks infected cells into this phase (Goh, Rogel et al. 1998). Since, as mentioned earlier, the average life span of HIV infected $CD4^+$ cells is about 24-48 hours, locking the cell in the G2 phase might be the way HIV has developed to maximize its reproductive efforts.

The final function of Vpr may be related to its first. It has been shown that Vpr faintly induces an increase in viral transcription. This observation would be consistent with the increased viral transcription seen during the G2 phase. However, it has been suggested that Vpr may directly interact with host cell transcription factors (Freed and Martin 2001).

The 17kd, 81 amino acid, integral membrane protein, Vpu, participates in two main activities. First the C-terminal cytoplasmic region of Vpu interacts with CD4 downregulating it on the surface of the cell (Chen, Gandhi et al. 1996). Degradation of CD4 is believed to follow a ubiquitin-dependent-proteasome pathway because after binding CD4, Vpu recruits TrCP, a member of a E3 ubiquitin ligase complex. (Bour, Perrin et al. 2001) Down regulation of CD4 serves two purposes. First, by reducing the primary receptor for HIV, infected cells are less likely to get infected again, thus increasing the number of cells that will become infected. Second, the chances that SU will interact with CD4 on its own envelope are decreased.

A side effect of Vpu binding TrCP is that there is a decrease in the activity of NF-κB. (Bour, Perrin et al. 2001) The Vpu interacts with TrCP without being degraded itself, which is unusual. This unusual interaction is believed to keep TrCP-dependent degradation of phosphorylated IκB-α during tumor necrosis factor (TNF)-α stimulation. Degradation of IκB-α releases NF-κB allowing it to act as a transcription factor. The lack of NF-κB transcription factor activity results in a decrease in transcription of the HIV-1 genes (Bour, Perrin et al. 2001). There are many immune response elements that are upregulated by NF-κB such as chemokines and cytokines, which HIV may be targeting instead.

The second activity associated with Vpu involves the stimulation of virion release. Vpu defective mutants show significant increases in the number of particles

remaining attached to the host cell membrane. (Lamb and Pinto 1997) Furthermore, it has been shown that Vpu is not required for efficient particle release in dividing cells but is essential for release in non-dividing cells, such as those locked in the G2 phase. (Deora and Ratner 2001)

The final accessory protein is the one I find most fascinating: Nef, which stands for **n**egative **ef**fect. The 27 kd, 206 amino acid, Nef protein is expressed early in the HIV life cycle and accounts for 80% of early RNA transcription (reviewed in (Marsh 1999)). Scientists originally believed Nef might play a role in the clinical latency seen in HIV infected patients. They believed this because in some cell lines it was shown that cells infected with Nef mutants replicated the virus faster than cells infected with wild type HIV. Others quickly refuted that observation and now experiments show that Nef has no effect or only offers a very moderate increase in replication *in vitro*.

Several activities have been credited to Nef. First like Vpu Nef can down regulate the expression of CD4 on the surface of cells. Nef accomplishes this task by increasing the endocytosis of CD4 in clathrin-coated pits. Nef has been shown to interact with both CD4 and the AP-2 adapter complex, which is responsible for recruiting membrane proteins to these pits for endocytosis (reviewed in (Arora, Fredericksen et al. 2002)).

Second Nef can also down regulate the expression of major histocompatibility complex class I (MHC I) on the surface of cells up to 300 fold (Collins, Chen et al. 1998). The MHC-I molecule, which will be discussed in detail in the next chapter, is responsible for presenting viral epitopes to the T-cell receptor on CD8 positive cells. Down regulation of MHC-I would decrease the immune system's ability to identify infected cells. The exact mechanism for MHC-I down regulation is still not fully deduced; what is known is that Nef interacts with PACS-1 (phosphofurin acidic cluster

sorting protein-1) and proceeds to endocytose MHC-1 through a PI3K-Regulated ARF6 Endocytic Pathway (Blagoveshchenskaya, Thomas et al. 2002).

Nef has also been shown to upregulate FasL. This is accomplished through a cascade of events that is triggered by Nef interacting with the T cell receptor ζ chain (reviewed in (Fackler and Baur 2002)). The up regulation of FasL may lead to the apoptosis of CTLs specific for HIV. This apoptotic event is triggered when Fas on CTLs bind the FasL on the infected CD4 cell. Nef is also responsible for minimizing apoptosis by cis acting Fas/FasL binding on the same infected cell by inhibiting the activation of apoptosis signaling kinases 1 (ASK1).

The HIV genome encodes for only 15 proteins that have evolved to ensure the reproduction of the virus. These proteins display complicated characteristics that allow for the reverse transcription of the RNA genome, the nuclear import and integration of the proviral DNA and the export of unprocessed RNA. The virus has evolved methods to increase infectivity while decreasing exposure to the immune system. The HIV pathogen presents a formidable opponent for the development of a vaccine.

Overview of the Immune Response

Our body's defensives can be divided into two general areas, innate immunity and specific immunity. The innate immunity is fairly non-specific and includes barriers such as your skin, the acidic environment of your stomach, tears, saliva and mucous membranes lining your respiratory and gastrointestinal tract. When pathogens get past the innate immune system they encounter your specific immune system, which must first identify the intruder before unleashing a counter attack consisting primarily of antibodies, which bind to pathogens outside cells, and cytotoxic T-lymphocytes (CTL), which destroy cells that have been infected by the pathogen.

The innate immune system response is effective against most pathogens the body comes across. However, since it is non-specific, there is little need to elaborate more on it because the goal of this work was the creation of specific vaccines. The specific immune system can be subdivided into two types of responses; the humoral and the cell-mediated. Furthermore, the specific immune system can be looked at in terms of the location of the response; whether it is systemic or mucosal.

Aside from the location and type of response, the immune system functions differently if it is responding to a primary infection or secondary infection. The primary response, which results from the initial exposure to an antigen, is mediated by naïve lymphocytes (B and/or T). In mice a primary response peaks 10-17 days post infection and is of relatively low magnitude and short duration. For humans the peak response can take longer. Furthermore, the primary immune response is characterized by the

production of antibodies from the immunoglobulin M class. (Antibody classes are discussed later.)

The second time the immune system sees the antigen, or after chronic infection, the immune system responds principally with memory lymphocytes. In mice the secondary response peaks after 2-7 days and has a much higher magnitude and longer duration compared to the primary response. The production of antibody from the immunoglobulin G class is the overwhelming antibody response observed. The goal of a vaccine is to ensure that when the actual antigen is seen the immune system treats it like a second encounter. In other words, the body has been prepared to more quickly counter the effects of the infection.

B-CELL MATURATION

Humoral immunity refers to the interaction of B-cells with pathogens, which leads to their proliferation and differentiation into plasma cells that secrete antibodies and form memory B-cells. B-cells begin in the bone marrow as lymphoid stem cells, and when given the right environment, which includes the cytokine IL-7 and direct interaction with stromal cells, they can differentiate into mature B-cells that express on their membrane a unique antigen-binding receptor. During maturation the B-cells go through a process of negative selection to insure that the antigen-binding receptor does not recognize self-antigens. This phase of maturation is designated as the antigen-independent phase.

After maturation, the mature B-cell, now termed naïve, is released into the periphery to circulate in the blood and lymphatic fluids. The naïve B-cell has yet to be exposed to its particular antigen and if it does not encounter it within a few weeks it will die by apoptosis. However, if the B-cell comes in contact with the antigen in the presence of an appropriate T-helper cell response, the B-cell becomes active. The active

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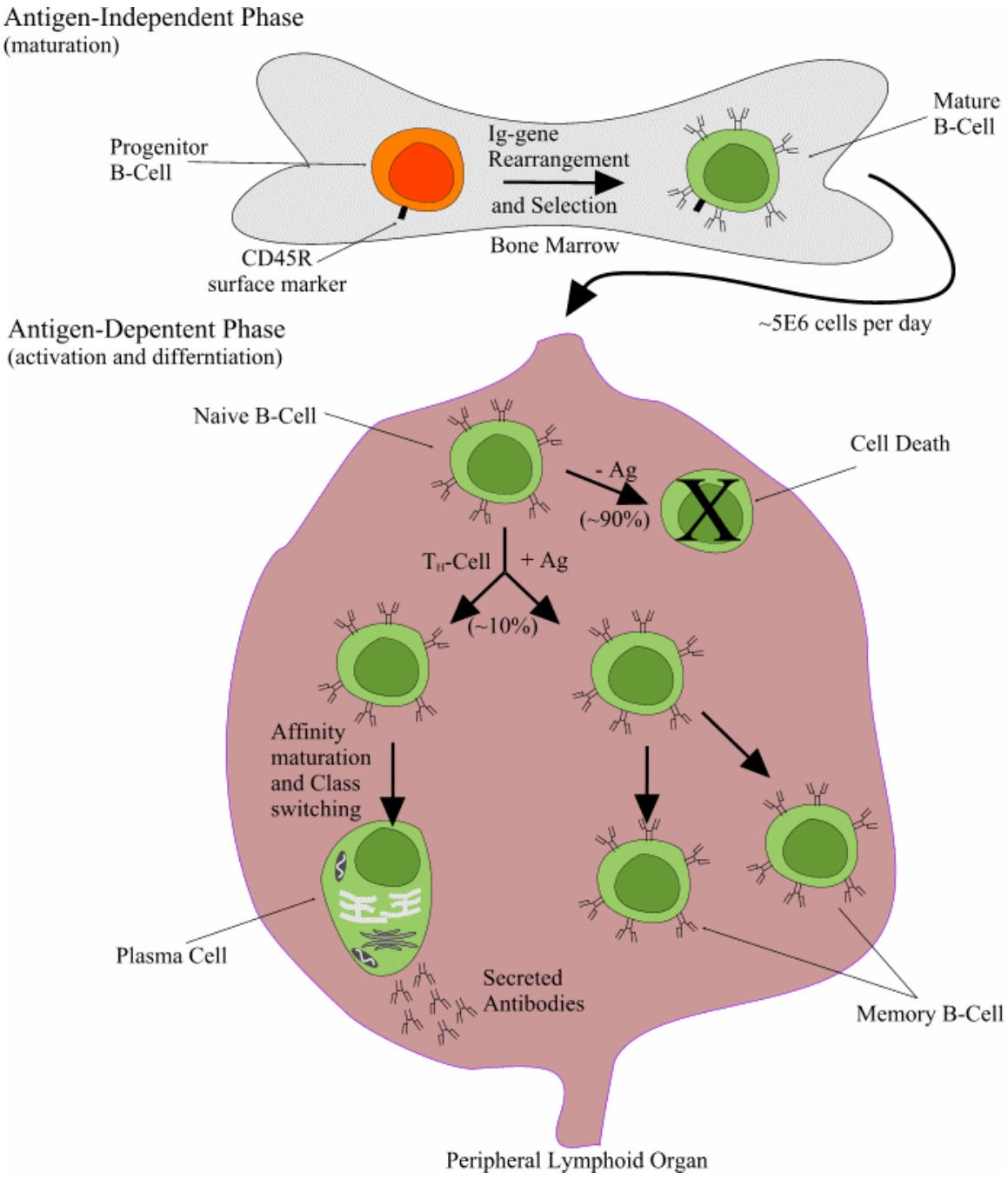


Figure 3.1 Schematic depiction of the B-cell maturation and differentiation process.

Adapted from Kuby Immunology, page 270 (Goldsby, Kindt et al. 2000).

B-cells go through a process of class switching and differentiation that results in plasma cells that can secrete antibodies and memory B-cells that can respond to future infections. This stage of B-cell differentiation has been appropriately referred to as the antigen-dependent phase (Figure 3.1).

TYPES OF ANTIGEN

The antigen recognized by naïve B-cells can be of protein, polysaccharide or lipid origin. The antigen can be a sequence of amino acids or a non-sequential motif. B-cell antigens can be categorized by their need for T_H cell contact. As seen in mice, there are two types of antigen; a few antigens fall into the category of thymus-independent (TI) antigens, while the majority of antigens are classified as thymus-dependent (TD) antigens. The TI antigens can further be divided into type 1 (TI-1) antigens and type 2 (TI-2) antigens.

As the name suggests TI antigens do not require direct contact with T_H cells. TI-1 antigens, such as the bacterial wall components lipopolysaccharide (LPS), peptido-glycan, and porin, are mitogens that cause the polyclonal activation of B-cells into differentiated antibody-secreting plasma cells regardless of their antigenic specificity. Not only are TI-1 antigens not bound by T_H-cell contact but they also do not require any cytokines secreted by the T_H-cells. TI-2 antigens recognized by B-cells, on the other hand, do require the B-cell to be in proximity to cytokines secreted by T_H-cells to trigger class switching. These types of antigen are not polyclonal activators and can only activate mature B-cells. TI-2 antigens are generally repeating polymers that are also found as part of the bacterial wall.

TD antigens are antigens that have been internalized by the B-cell receptor (BCR), processed through the endocytic pathway, and presented in the class II major histocompatibility complex (MHC-II). Cross-linking of the membrane bound immunoglobulin (IgM) by the antigen results in the phosphorylation of the Igα/β immunoreceptor tyrosine-based activation motif (ITAM). This initiates an effective intracellular signal, which provides the first signal for activation. This signal leads to an increase in both MHC-II expression and the expression of the co-stimulatory molecule B7. The T-cell receptor (TCR) on $CD4^+$ T cells recognizes the antigen, which triggers the activation of the T_H-cell. The activation of T_H-cell results in the secretion of cytokines, such as interleukin 2 (IL-2), IL-4, IL-5, and others, and in the expression of CD40L, which is a member of the tumor necrosis factor receptor family. CD40L interacts with a member of the tumor necrosis factor surface protein family known as CD40 on the B-cell. The CD40L/CD40 cross-linking triggers a second signal in the B-cell. This signal is amplified when the co-stimulatory molecule B7 interacts with CD28 on the T_H-cell. The amplified signal drives the naïve B-cell from the G_0 phase into the G_1 phase of the cell cycle pathway resulting in an activated B-cell.

Activation of the B-cells causes the upregulation of cytokine receptors on the surface of the cells. The cytokines released by bound T_H-cells, or released by other cytokine producing cells in the area, interact with the cytokine receptors on the B-cell. This event directs the B-cell to proliferate and differentiate into plasma cells and memory B-cells (Figure 3.2).

There are several differentiations to plasma cells possible depending on the type of cytokines present. A differentiating B-cell in the presence of interferon gamma (INF-γ) gives rise to plasma cells secreting immunoglobulin G2a (IgG2a) or IgG3. Differentiation in the presence of transforming growth factor-β (TGF-β) triggers the

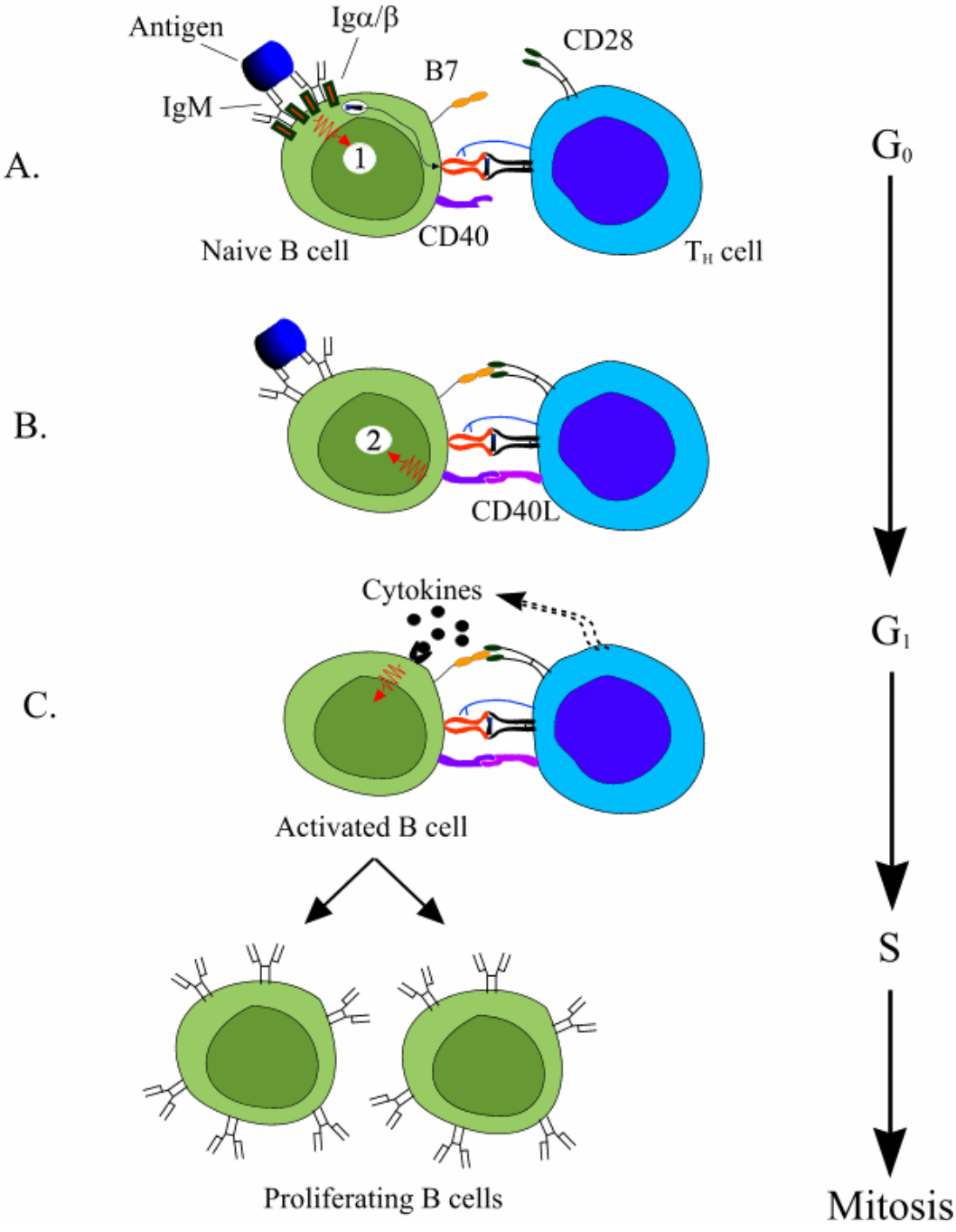


Figure 3.2 Schematic depiction of the activation and differentiation of B-cells by a TD antigen.

Adapted from Kuby Immunology, page 282 (Goldsby, Kindt et al. 2000).

creation of plasma cells releasing IgG2b or IgA. Plasma cells secreting antibodies IgE and IgG1 result from proliferation of B-cells in the presence of IL-4. The cytokines IL-2, IL-4, and IL-5 generate plasma cells secreting IgM (Figure 3.3).

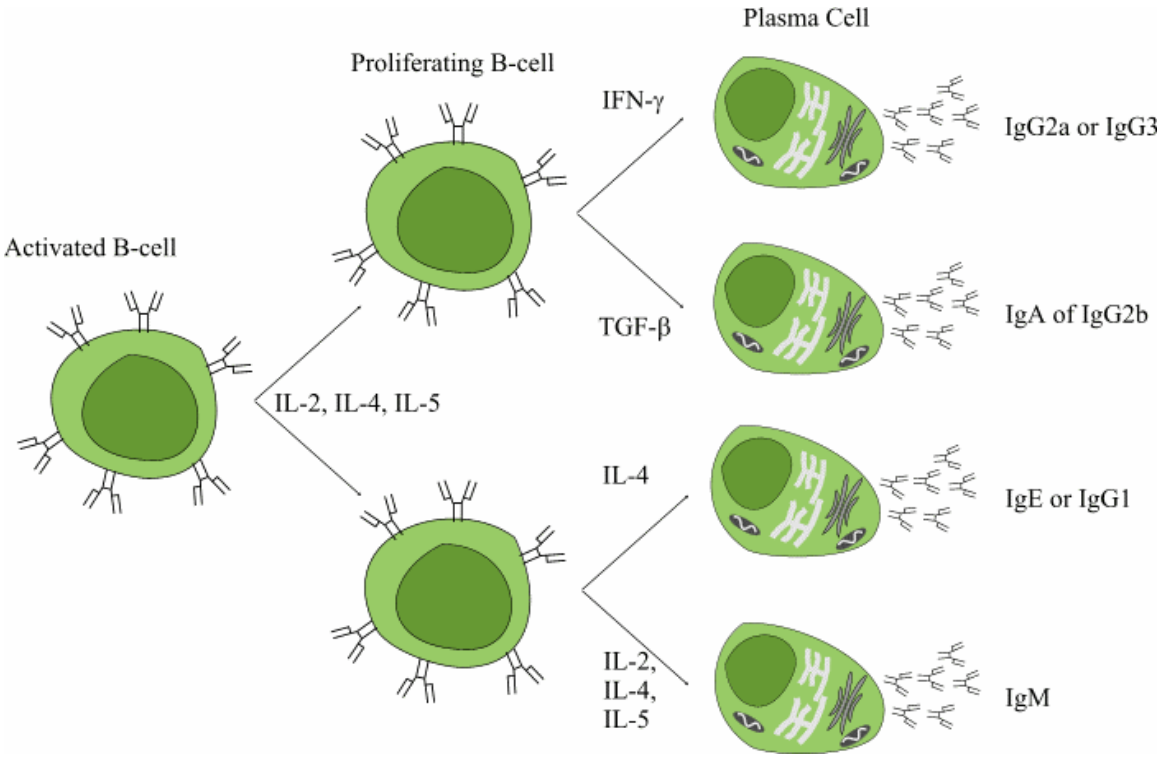


Figure 3.3 Schematic depiction of the cytokine driven B-cell differentiation.

B-cells differentiate into plasma cells secreting different immunoglobulins. Adapted from Kuby Immunology, page 295 (Goldsby, Kindt et al. 2000).

IMMUNOGLOBULIN CLASSES

Immunoglobulins, or antibodies, are present on the surface of B-cells or are secreted by the plasma cells. Ig has a basic structure of two homogeneous light chains of about 220 amino acids attached by disulfide bonds to two homogeneous heavy chains of about 440 amino acids. Each light and heavy chain contains two domains, one C-terminal constant domain known as C_L or C_H, respectively, and one N-terminal variable domain termed V_L and V_H respectively. Both V_L and V_H include regions of concentrated sequence differences that have been termed hypervariable regions. These hypervariable regions form two antigen-binding sites on the N-terminals of the Ig molecule (Figure 3.4).

Antibodies interact with antigens in a non-covalent manner triggering several outcomes. First after binding the antigen, the C_H regions of the antibody can interact with receptors on phagocytic cells, causing receptor mediated phagocytosis of the antigen, also known as opsonization. Second, after binding the antigen, the C_H regions of the antibody can trigger the complement pathway; which results in the formation of a membrane attack complex that can lysis invading cells. Third, when an antibody binds an antigen on the surface of an infected cell, for example the antigen can be part of a budding virus particle, the CH regions can call in natural killer cells to lyse the infected cell. This process is known as antibody-dependent cell-mediated cytotoxicity. Finally, the actual binding of the antibody to the antigen could neutralize an important activity of the invader.

As alluded to above there are several classes of Igs. The classes are distinguished from one another by differences in their C_H domain. The Igs are divided into the classes, IgG, IgM, IgA, IgE and IgD. Minor sequence variations in the C_H domain of IgG and

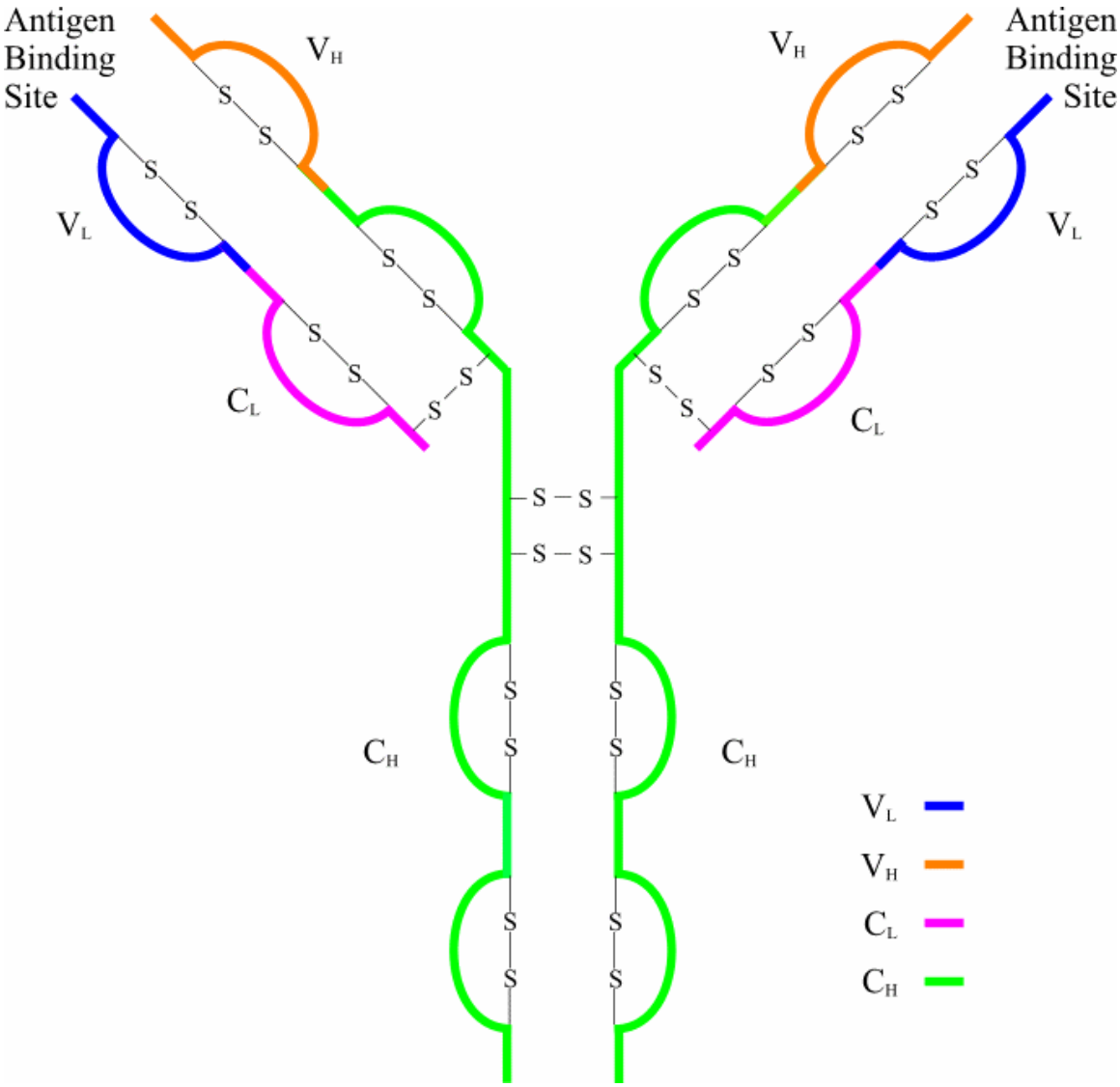


Figure 3.4 Schematic depiction of the immunoglobulin structure.

The structure consists of two homogeneous light chains attached by disulfide bounds to two homogeneous heavy chains. Each light and heavy chain contains two domains, one C-terminal constant domain known as C_L or C_H, respectively, and one N-terminal variable domain termed V_L and V_H respectively. The coming together of the VL and VH domains forms the antigen binding site. Adapted from Kuby Immunology, page 84 (Goldsby, Kindt et al. 2000).

IgA allow them to be categorized further into subclasses. In humans the subclasses for IgG are IgG1, IgG2, IgG3, and IgG4, while in mice they are termed IgG1, IgG2a, IgG2b, and IgG3. IgA in humans can be broken down into IgA1 and IgA2, while in mice no subdivision of IgA is observed. Because the distinguishing characteristic of an Ig class is the variations in the C_H regions, class effector functions may differ.

IgG is secreted from plasma cells as a monomer. It is the most abundant Ig class found in the serum, accounting for about 80% of the total. As for the isotypes, the concentration in serum has this relative intensity: IgG1 > IgG2 > IgG3 > IgG4. IgG1, IgG2 and IgG4 have half-lives of close to 23 days while IgG3 has a half life of only 8 days. The antibody effector functions also differ between subclasses. In decreasing efficiency IgG3, IgG1 and IgG2 activate the complement pathway. IgG1 and IgG3 have a high affinity for receptors on phagocytic cells and thus effectively initiate opsonization. Furthermore, IgG1, IgG3 and IgG4 play a role in the protection of the fetus because of their unique ability to cross the placenta.

The immunoglobulin produced first in a primary response is IgM. IgM accounts for somewhere between 5-10% of total serum antibodies in humans under normal conditions. It is seen in the monomeric form attached to the membrane of immature and mature B-cells. IgM secreted by plasma cells forms a pentamer that has a half life of 5 days. The pentamer results from disulfide bonds between C_H and the incorporation of an additional polypeptide, known as the J chain. The J chain gives the secreted IgM the ability to cross the epithelial lining between the systemic and mucosal areas of the body. Thus the pentamer form of IgM can be found in external fluids, such as saliva, tears, breast milk and mucus. IgM is most noted for its abilities to trigger the complement pathway and facilitate the opsonization of large molecules.

Although IgM can be secreted, the most abundant immunoglobulin class found in the external fluids mentioned above is IgA. In serum IgA accounts for 10%-15% of the total antibodies and is generally found as a monomer, but it can also form multimers. In external fluids IgA is most often found as a dimer bound also to a J chain and an additional molecule known as the secretory component. The secretory component is incorporated during the transport of IgA across the epithelial cell layer. IgA activates the complement via an alternative pathway compared to the classical pathway used by IgG and IgM. IgA has been shown to be effective in preventing the attachment of some antigens to mucosal cells.

The immunoglobulin class, IgE has a half-life of 2.5 days and accounts for less than 0.002% of serum antibodies. IgE is believed to be important in the removal of parasites from the gut and respiratory tract, but is most known for its ability to trigger hypersensitivity reactions. The C_H domain of IgE can bind a receptor on mast cells and basophils known as FcεRI. Binding prompts degranulation and cytokine production resulting in allergic reactions with symptoms such as hives, asthma, hay fever, and in the worst-case scenario anaphylactic shock.

IgD, the final class of immunoglobulins, has a half-life of 3 days and accounts for less than 0.2% of serum antibodies. IgD can be found bound to the membrane of mature B-cells, but not immature ones. To date, no antibody effector function has been established for this class of immunoglobulin. Since it is one of the only two antibodies expressed on naïve mature B-cells it is believe to play a role in B-cell activation.

T-CELL MATURATION

While B-cell maturation occurs in the bone marrow, T-cell maturation transpires in the thymus. When progenitor T-cells arrive in the thymus they do not carry the characteristic T-cell markers such as the TCR and accessory molecules CD4 or CD8, the most important of which is the TCR. Over a three-week period, the progenitor T-cell creates a TCR and verifies its functionality.

The TCR is a transmembrane heterodimer that is held together by disulfide bridges and consists of either an α and β chain or a δ and γ chain. The two types of TCR are aptly termed $\alpha\beta$ TCR and $\gamma\delta$ TCR. The $\alpha\beta$ TCR accounts for over 95% of the T-cell receptor molecules in adults. Like antibodies the TCR chains contain both a variable domain and a constant domain. Through a random process of gene rearrangement, which is beyond the scope of this writing, the T-cells create a pool of TCRs with an antigenic diversity potentially exceeding 10^{15} for $\alpha\beta$ TCR and 10^{18} for $\delta\gamma$ TCR. Also like the membrane bound antibodies, the cytoplasmic tail of the TCR is small and thus it associates with other proteins, CD3, forming the TCR-CD3 complex. CD3 are a group of proteins that have longer cytoplasmic tails that contain the ITAM motif and are necessary for intracellular signaling (Figure 3.5). Unlike antibodies the TCR is confined to recognizing antigens presented in one of the two classes of MHC molecules, MHC-I and MHC-II.

Since the gene rearrangement is a fairly random process (for review see chapter 9 in (Goldsby, Kindt et al. 2000)), not all TCR created will have the ability to recognize the MHC and some might recognize self antigens. To ensure that the TCR recognizes antigen presented in the MHC molecules the T-cell undergoes a process known as positive selection. This takes place in the cortical region of the thymus and involves the cortical epithelial cells. Immature T-cells that interact with the MHC molecules on these

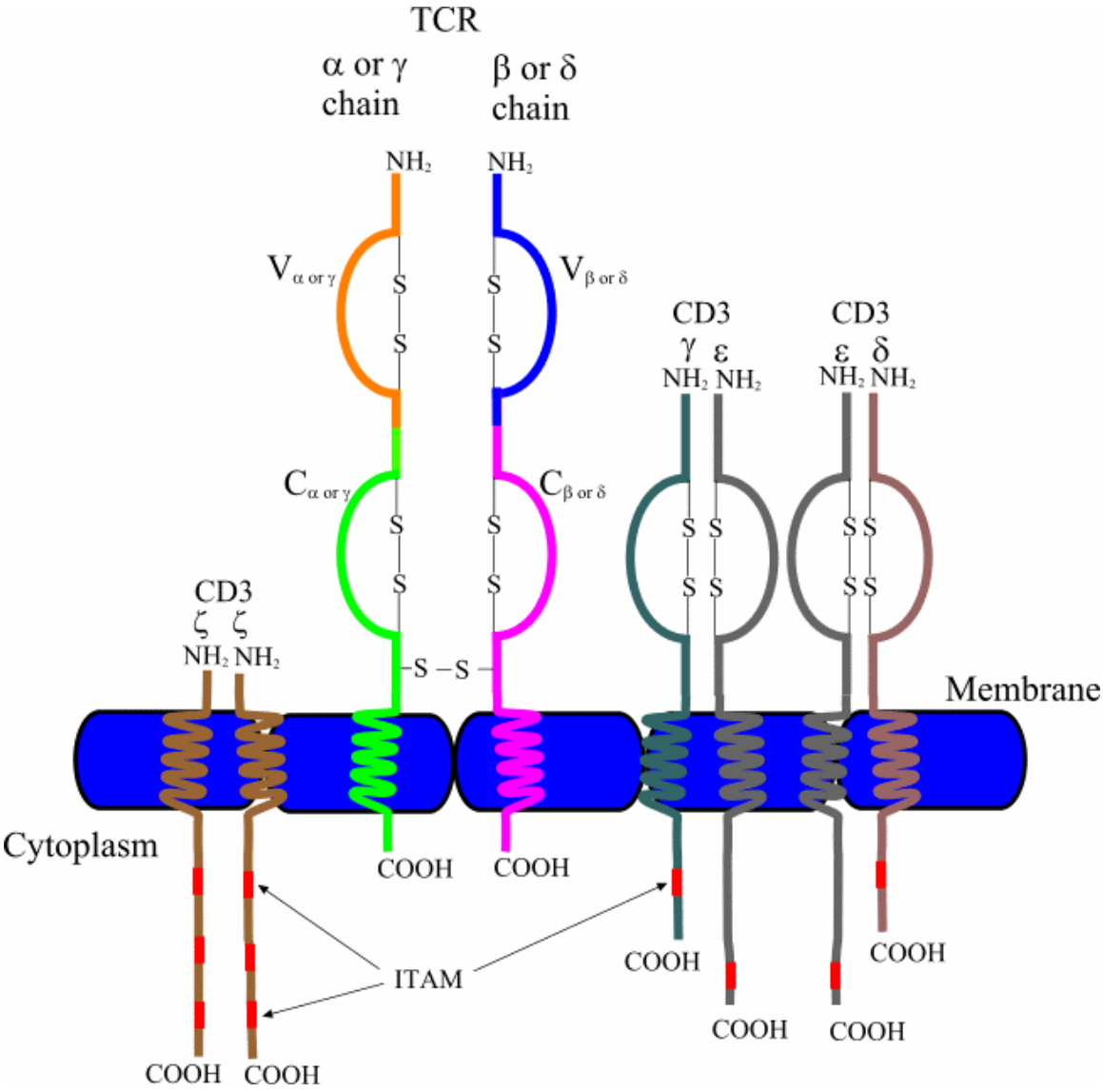


Figure 3.5 Schematic depiction of the T-cell Receptor (TCR) and CD3 complex.

Adapted from Kuby Immunology, page 227 (Goldsby, Kindt et al. 2000).

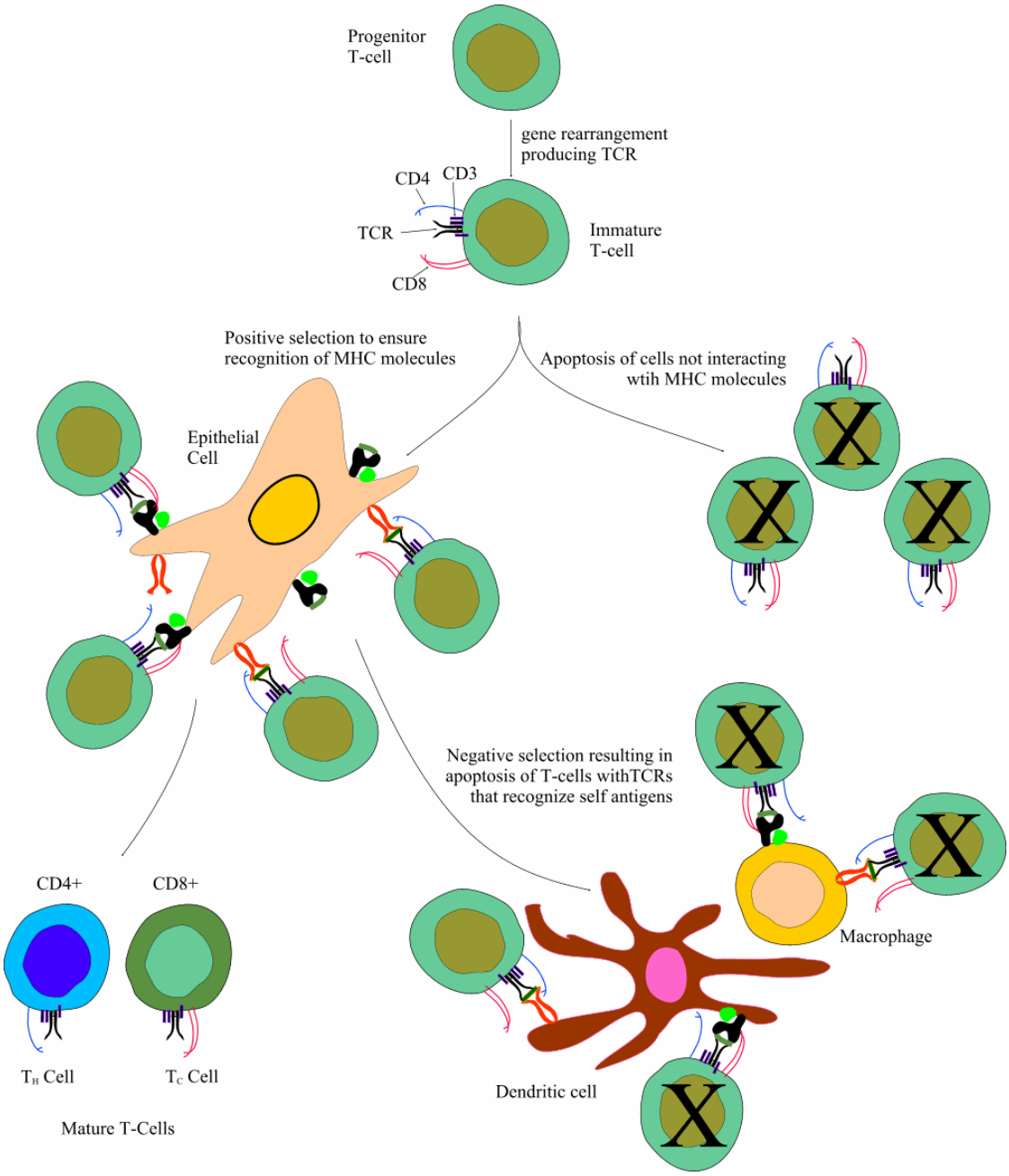


Figure 3.6 Schematic depiction of positive and negative selection.

T-cell selection process ensuring TCRs that recognize self MHC molecules but not self antigens. Adapted from Kuby Immunology, page 244 (Goldsby, Kindt et al. 2000).

cells are believed to receive a signal that prevents apoptosis. T-cells that do not receive this signal initiate programmed cell death. Next, to prevent the destruction of cells presenting normal cell antigens, the T-cell goes through a procedure known as negative selection. Negative selection involves interactions with the antigen presenting cells in the medulla region of the thymus. It is believed that T-cells that bind antigen presented in the MHC molecules on the dendritic cells and macrophages in this region of the thymus receive a signal triggering programmed cell death. It is estimated that less that 5% of T-cells pass the selection process and are allowed to leave the thymus (Figure 3.6).

After leaving the thymus the T-cell is considered mature because it has a functional TCR; however, it is still referred to as naïve because it has not yet encountered antigen. The naïve T-cell continually circulates through the bloodstream, spleen and lympathic vessels, such as lymph nodes and Peyer's patches. Any given naïve T-cell is believed to circulate through these areas every 12-24 hours. The circulation of the T-cell increases the chances that it will encounter a cell presenting an antigen it recognizes. If the T-cell does not encounter antigen it is believed to trigger apoptosis after 5-7 weeks of circulation. However, if the naïve T-cell detects an antigen it becomes active and proliferates into effector T-cells and memory T-cells.

$CD4^+$ T-CELL CO-STIMULATION REQUIREMENTS

The activation process differs if the naïve T-cell is $CD4^+$ or $CD8^+$ and if the TCR is $\alpha\beta$ or $\gamma\delta$. Since the majority of TCR are $\alpha\beta$, that will be the pathway described here. As mentioned earlier $CD4^+$ cells exclusively identify antigen presented in the MHC-II molecule and are almost exclusively T_H-cells. The initial interaction of the TCR with the antigen in the MHC-II molecule 'results in the upregulation of cell surface molecules

such as CD28, IL-2 receptor (IL-2R) β and IL-2Rγ, and in the induction of surface expression of CD40 ligand (CD40L), Ox40, 41BB, IL-2Rα and other' molecules (Whitmire and Ahmed 2000). In addition to the interaction with the MHC-II molecule, the activation of T_H-cells requires one or more co-stimulation signals.

Potential co-stimulation signals result from the interaction of CD40 with CD40L, the interaction of CD28 with B7, or the interaction of Ox40 with the Ox40L. Using CD40L-deficient mice infected with lymphocytic choriomeningitis virus (LCMV), recombinant adenovirus, or murine γ-herpesvirus 68 (MHV-68) it has been shown that the $CD4^+$ response is reduced by greater than 5-10 fold (reviewed in (Whitmire and Ahmed 2000)). A similar reduced $CD4^+$ response is seen in Ox40-deficient mice infected with LCMV and influenza virus (reviewed in (Whitmire and Ahmed 2000)). Also when mice treated with anti-B7 molecules to block the CD28/B7 interaction, the mice demonstrated impaired $CD4^+$ responses when infected with LCMV (reviewed in (Whitmire and Ahmed 2000)). However, mice unable to create the 41BB-41BBL (ligand) interaction, which were infected with LCMV or influenza virus mounted a $CD4^+$ response similar to the wild type mice (reviewed in (Whitmire and Ahmed 2000)). This implies that not all molecules upregulated during the initial interaction with antigen presented in the MHC-II molecule are important in activation.

$CD8^+$ T-CELL CO-STIMULATION REQUIREMENTS

The activation of naïve $CD8^+$ T cells (or T_C-cell) differs from the $CD4^+$ T-cells. As mentioned earlier the $CD8^+$ T cells recognize antigen presented in the MHC-I molecule. T_C- cells can be distinguished by their requirement for T_H-cell responses. In mice it has been shown that infections with acute LCMV, Sendai virus, vaccinia virus,

influenza virus, Ectromelia virus, or MHV-68 can induce T_C-cell responses in the absence of $CD4^+$ cells, whereas mice infected with Adenovirus, Japanese encephalitis virus, or Herpes simplex virus require $CD4^+$ cells to become active (reviewed in (Whitmire and Ahmed 2000)).

Like $CD4^+$ cells the recognition of antigen triggers 'the upregulation of cell surface molecules such as CD28, IL-2 receptor (IL-2R) β and IL-2Rγ, and in the induction of surface expression of CD40 ligand (CD40L), Ox40, 41BB, IL-2Rα and others (Whitmire and Ahmed 2000). However, unlike the $CD4^+$ T-cells a co-simulation signal requirement depends on the antigen recognized. It has been shown that T_H-cell independent activation does not require CD40/CD40L interaction or Ox40/Ox40L interaction. The requirement for the CD28-B7 interaction depends on the virus. T_H-cell independent activation requires CD28/B7 interaction when mice are infected with vaccinia virus, VSV, and influenza (subtype H_1N_1), but is not required during acute LCMV infection or infection with influenza (subtype H_2N_2). Whereas in $CD4^+$ T cell activation the 41BB/41BBL interaction was not required, it appears to be necessary in both T_H-cell independent and T_H-cell dependent activation of $CD8^+$ T-cells. T_H-cell dependent activation also showed a requirement for the CD40/CD40L and the CD28/B7 interactions (reviewed in (Whitmire and Ahmed 2000)).

Although the CD40/CD40L interaction was not necessary for the activation of T_C-cells during T-cell independent infections, it has been demonstrated that CD40 deficient mice generated 10 fold fewer memory cells after LCMV infection compared to the wild type. If these mice are reinfected with LCMV they are able to mount a memory cell response, which implies that, although there were less memory cells, they were still functional. Thus the CD40/CD40L response is probably involved in inhibiting portions of the death phase of the T-cell cycle (reviewed in (Whitmire and Ahmed 2000)).

IL-2 REQUIREMENT

Another signal unrelated to intercellular interactions is necessary to activate both the T_H-cells and T_C-cells. As mentioned above, the expression of a high affinity IL-2R is induced when the TCR first recognizes the antigen. Also upon antigen recognition, both the T_H-cells, especially T_H-1, and to a lesser extent the T_C-cells begin secreting IL-2. The IL-2 secreted by these cells can then attach to the IL-2R on their own surface or the IL-2R on T-cells in the area. This interaction provides the final signal necessary to induce the cascade of events that results in sustained T-cell proliferation and differentiation.

EFFECTOR CELLS

After the initial signal and, when necessary, the co-stimulation signal are produced, the now active T_H-cell or T_C-cell proliferates and differentiates into effector and memory cells. There are two subsets of effector T_H-cells that are termed T_H1 and T_H2, and there is only one type of effector T_C-cells, known as CTL. In both cases the initial interaction of an effector cell with professional antigen presenting cells (discussed later) or potential target cells is weak. This weak interaction gives the TCR time to distinguish the antigen presented. If the TCR is not specific for the antigen, then the effector cell disengages from the other cell. If, however, the TCR is specific, then the cellular interactions increase due to an increase in the affinity of the leukocyte function-associated molecule 1 (LFA-1) on the effector cell for the intracellular adhesion molecule-1 (ICAM-1) on the target cell. Without a co-stimulation signal the effector cells proceed to carry out their respective functions.

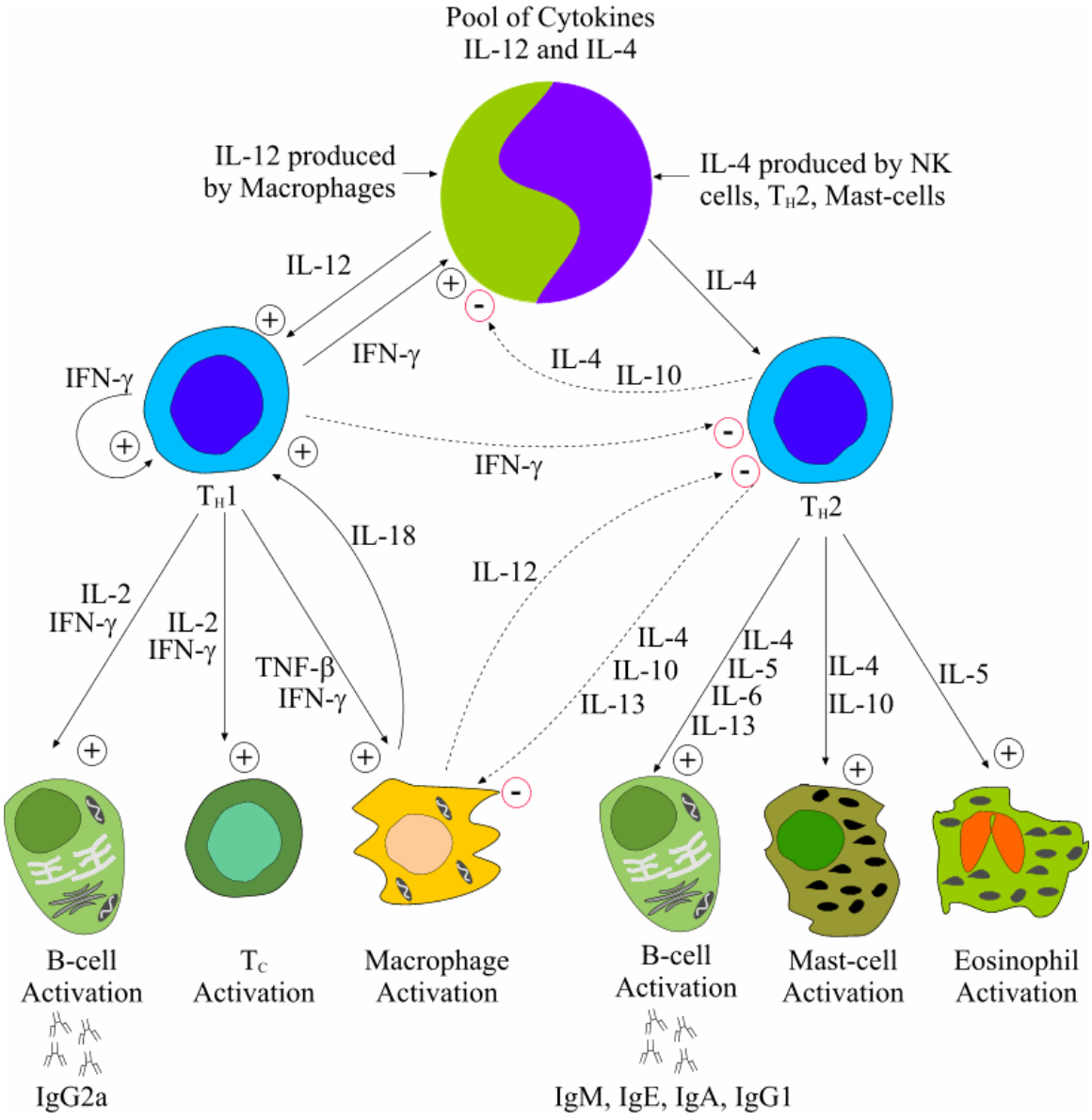


Figure 3.7 Schematic depiction of the activity of cytokines released by T helper cells.

Adapted from Kuby Immunology, page 320 (Goldsby, Kindt et al. 2000).

Both subsets of T_H-cells secrete the cytokine IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The T_H1 effector cells specifically secrete cytokines IL-2, interferon (INF)-γ, and TNF-β. T_H1 effector cell cytokines increase the B-cell production of IgG isotypes that initiate complement, most notably IgG2a in mice. They also activate macrophages, activate T_C-cells, participate in the delayed-type hypersensitivity and promote the inflammatory response. The T_H2 effector cells specifically release cytokines IL-4, IL-5, IL-10, and IL-13. These cytokines prompt B-cells to secrete IgM, IgE and IgG1. A ratio of IgG2a/IgG1 in mice is a common way of determining the relative strengths of the T_H1/T_H2 response. T_H2 cytokines also activate eosinophils and mast cells (Figure 3.7).

Effector CTLs secrete cytokines, in particular IFN-γ and TNF-β, but they serve a lytic function as well. These cells have two mechanisms for inducing the destruction of the target cells. One mechanism involves the Fas/Fas ligand (FASL) interaction, and the other involves the release of perforin and granzymes. Upon recognition of the antigen, CTLs upregulate the expression the membrane bound molecule FasL. When the FASL binds the Fas expressed on the target cell it triggers an apoptosis pathway through the activation of the FAS-associated death domain protein and caspase proteins (Krammer 2000).

A noticeable difference between naïve T_C-cells and CTLs is the increase in the number of granules in CTLs. These granules contain perforin and granzymes, which are then released, upon antigen detection, into the extracellular space between the CTL and target cell. The perforin molecules are exclusively found in the granules of CTLs and not in other granule containing cells such as basophils, eosinophils and neutrophils. These molecules insert themselves into the target cells' membrane, creating a perforin channel. The formation of the channel in itself does not cause lysis of the cell but instead allows

the entry of granzymes. Granzymes can also enter the cell via receptor-mediated endocytosis but require the perforin molecule to exit the endosome. There are four types of granzymes that have been identified in humans and nine types in mice. Inside the cell these granzymes are proteases that can trigger the caspase apoptosis pathway used by the Fas/FasL binding method and can cause mitochondria malfunctions that lead to necrosis (reviewed in (Krammer 2000)).

ANTIGEN PRESENTATION IN THE MHC-II MOLECULE

Presentation of peptides to $CD4^+$ cells in the MHC-II molecule follows a pathway known as the endocytic pathway. First the antigen is taken up by APC either through receptor-mediated endocytosis, pinocytosis, or phagocytosis. The antigen moves through several compartments with decreasing pHs and is finally degraded in the low pH (4.5-5.0) compartment known as the lysosome, which also contains hydrolytic enzymes. While the antigen is being processed, class II MHC molecules in association with the invariant chain (Ii) are transported from the ER to the Golgi complex. The class II MHC is translated directly into the ER. There it associates with the Ii to prevent it from binding peptides proceeding through the cytosolic pathway (described later). The MHC-II molecule and Ii leave the Golgi and enter the endocytic pathway. As the pH decreases, from compartment to compartment, the Ii is degraded leaving only a small portion of itself left in the antigen binding cleft. This portion is known as the class II-associated invariant chain peptide (CLIP) and is believed to prevent immature association of peptide with the binding site. At some point the compartment containing the degraded antigen fuses with the compartment containing the MHC-II molecule. CLIP is displaced and an

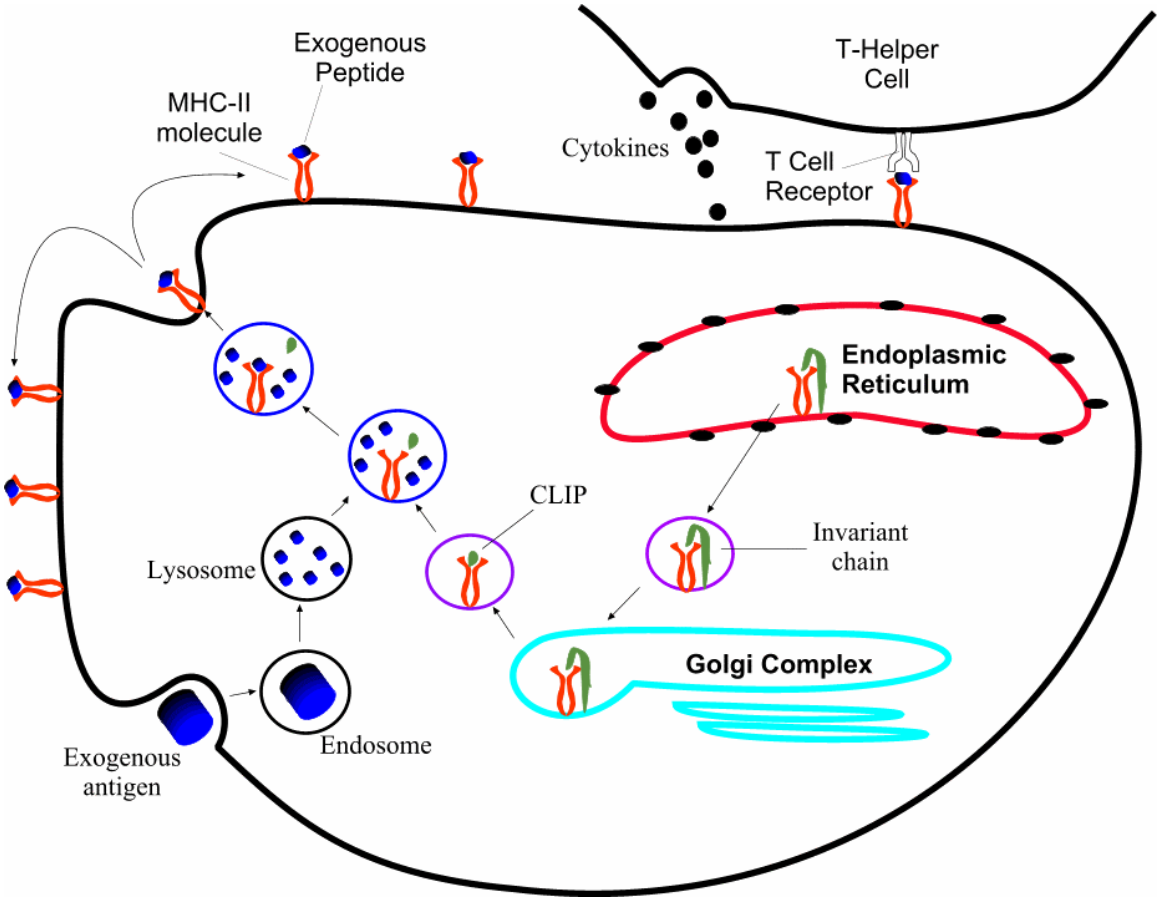


Figure 3.8 Schematic depiction of the endocytic pathway.

exogenous peptide takes its place. The MHC-II molecule is then exocytosed allowing it to present its peptide to the $CD4^+$ cells (Figure 3.8).

The MHC-II molecule is a heterodimer consisting of an α and β chain, which is expressed primarily on antigen-presenting cells (APC) such as macrophages, dendritic cells, and B-cells, but can also be found on the epithelial cells in the cortical region of the thymus. The α and β chain are both transmembrane proteins and associate via non-covalent interactions. Both proteins have two domains with the N-terminal membrane-distal domain referred to as 1, (α1 and β1) and the C-terminal membrane-proximal domain as 2, (α2 and β2). Interaction between α1 and β1 form the antigen-binding cleft, which holds the exogenous peptide for presentation to the T_H-cells.

The peptides found in MHC-II molecules are generally 13-18 amino acid residues in length. The MHC-II antigen binding cleft only interacts with 13 of the residues in the peptide. The cleft formed by the α1 and β1 domains is open and allows excess amino acid residues to hang over the sides. Peptides that bind are diverse in sequence but they often times have a hydrophobic or aromatic residue at the N-terminus and three hydrophobic residues in the middle. A proline has been found at the 2^{nd} residue in 30% of MHC-II peptides analyzed.

ANTIGEN PRESENTATION IN THE MHC-I MOLECULE

The peptide/antigen presented in MHC-I goes through a process known as the cytosolic pathway to get there. Proteins are tagged with ubiquitin and are sent to the proteasomes. The proteasome is a large multifunctional protease complex that chops the protein into smaller peptides. Peptides between the lengths of 8-12 amino acids that possess the appropriate residues are shuttled into the ER by the transporters associated

with antigen processing (TAP). TAP along with chaperones, like calnexin and tapasin, are responsible for loading the peptide into the groove on the MHC heavy chain. The assembled MHC I complex (MHC heavy chain, β_2-microglobulin (β_2M), and the peptide) leave the ER in a vesicle, which travels to the Golgi complex before being exocytosed into the extracellular environment. If CTL receptors recognize the peptide on the MHC it triggers the lytic functions of the CTL (Figure 3.9).

Unlike the MHC-II molecule the MHC-I molecule is ubiquitous and thus any cell the CTL comes in contact with is a potential target cell. The MHC-I molecule consists of the MHC heavy chain, β_2-microglobulin (β_2m), and the peptide (Figure 3.10). The MHC heavy chain is a transmembrane protein that contains three external domains known as α1, α2, and α3. Domains α1 and α2 come together in a manner that creates a groove that acts as the peptide-binding cleft.

The peptide binding cleft of MHC-I preferentially binds peptides with lengths of 8-10 amino acids; however, larger class I peptides are known (Jiang, Borthwick et al. 2002). The peptide binding cleft generally interacts with particular residues of the peptide at the N-terminal and C-terminal. These residues are designated as anchor residues and they are generally hydrophobic in nature. It has been shown that a given MHC-I allele can bind more than 2000 distinct peptides. There are an estimated 10^5 MHC allele molecules per cell. It has been suggested that a particular peptide needs to occupy as few as 100 MHC molecules to cause a peptide specific CTL response (Goldsby, Kindt et al. 2000)

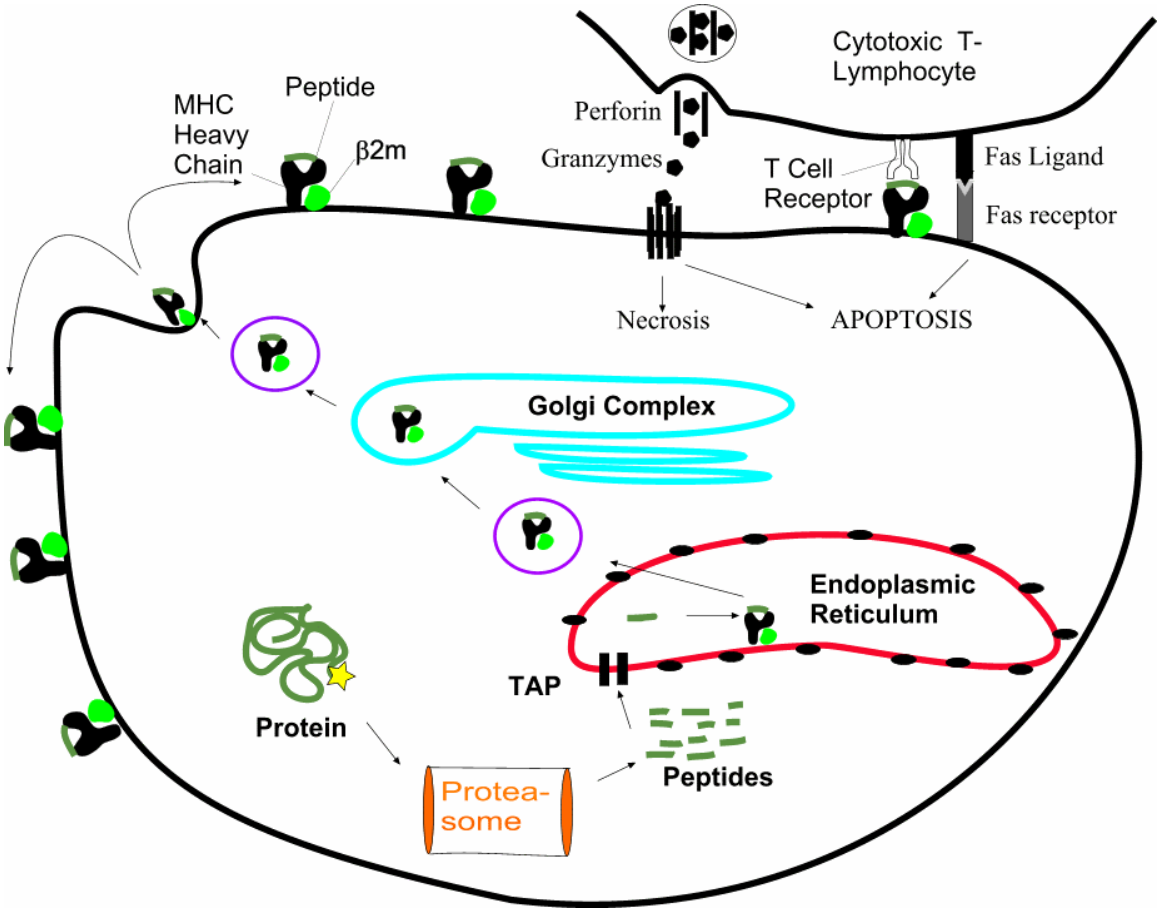


Figure 3.9 Schematic depiction of the cytosolic pathway

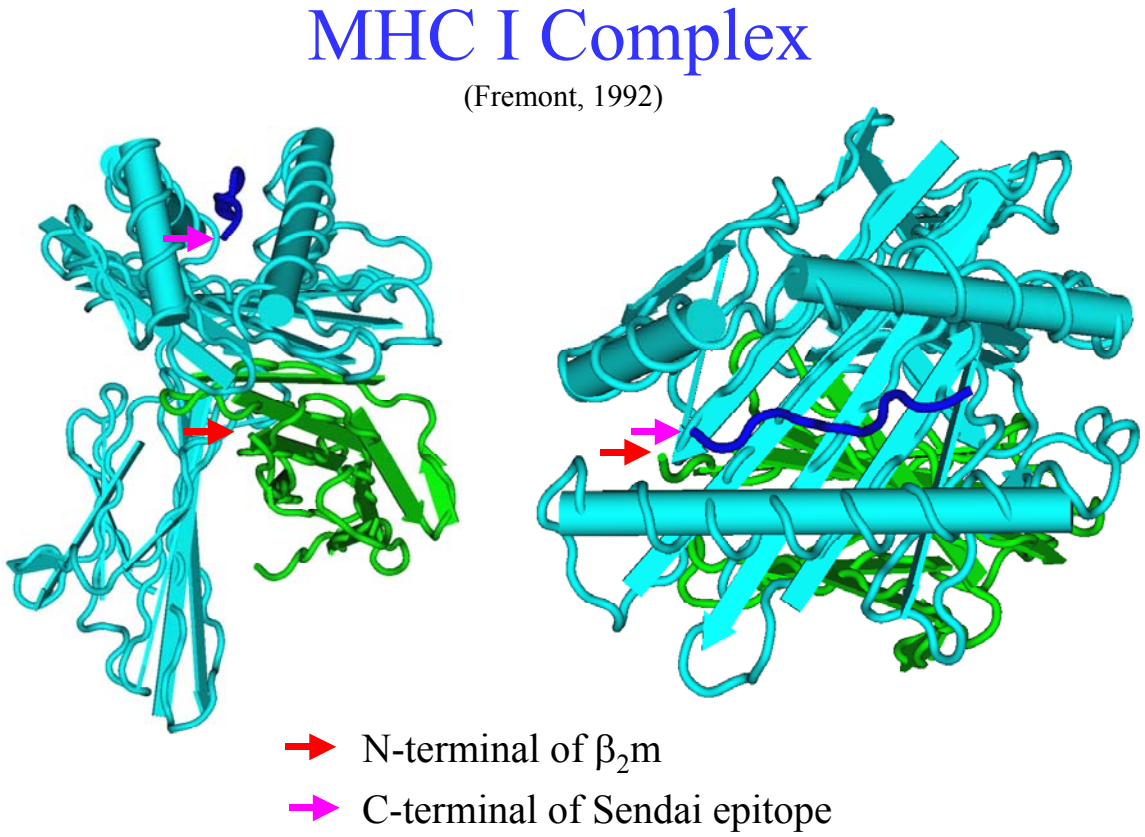


Figure 3.10 The structure of the MHC-I complex published by Fremont.

The MHC heavy chain is in light blue, the β_2-microglobulin (β_2m) in green and the Sendai peptide from the nucleoprotein amino acids 324-332 is in dark blue (Fremont, Mutsamura et al. 1992).

Unlike the MHC-I heavy chain, which has hundreds of alleles, as discussed above, β_2M is located on a different chromosome from the rest of the MHC molecules and is not considered polymorphic. This will be an important consideration as discussed in chapter 5 on Project Approach. β_2m contains no transmembrane anchor residues and associates with the heavy chain through non-covalent interactions. β_2m makes contact with all three external domains of the MHC heavy chain and provides structural support to the MHC-I complex as a whole. A type of tumor cell known as Daudi cells do not contain β_2m and are unable to present peptides in the class I molecule, illustrating the importance of β_2m molecule in the MHC-I complex.

MHC MOLECULE NOMENCLATURE

The molecules involved in the formation of both the MHC-I and MHC-II molecule are located on chromosome 6 in humans and chromosome 17 in mice. The MHC molecule in humans is known as HLA for human leukocyte antigen and in mice it is termed H-2. The molecules are then classified further based on the locus from which the gene was located. For humans the MHC-I molecules are designated as HLA-A, HLA-B, and HLA-C and in mice they are labeled H-2K and H-2D. The MHC-II molecule is termed HLA-DP, HLA-DQ, and HLA-DR in humans and in mice it is called H-2IA and H-2IE.

The loci that encode for the MHC molecule are highly polymorphic and thus many MHC gene forms exist. These different forms are known as alleles and in the population as a whole there are hundreds of different allelic variations of these MHC molecules. These MHC alleles can differ in DNA sequence up to 5%-10%, which results in gene products with similar yet unique structures. Any given individual is only likely to

express up to 6 variations of the MHC-I molecule and 12 variations of the MHC-II molecule. Most individuals inherit two sets of alleles, one set from each parent. A set of alleles is known as a haplotype. With mice there is a potential for inbreeding and thus haplotypes are often designated along with the loci region coding for the MHC molecule. A superscript in the MHC designation such as H-2^a, H-2^b, H-$2K^k$, or H-IA^d denotes the haplotype.

To obtain more information about the immune system functions covered in this chapter and those aspects that were not covered please refer to Kuby Immunology, from which this chapter borrowed heavily (Goldsby, Kindt et al. 2000).

Immune Response to HIV

In the last chapter, I presented an overview of the immune system; in this chapter I hope to provide a picture of the immune response specific for HIV. Understanding how the immune system naturally counteracts this viral infection will provide insight in to the development of an HIV vaccine. Also in this chapter I intend to highlight the mechanisms HIV uses to evade the immune response.

The first response to any viral infection comes from the cells in the innate immune system and the response to HIV is no different. 'The innate system includes macrophages, natural killer cells (NK cells), natural killer T cells (NK T), peripheral blood mononuclear cells, soluble factors produced by each of these cell types, and the complement system' (Redpath, Angulo et al. 2001). The relative contribution of these cells to HIV control is not fully understood. What is known is that activation of the innate immune system results in the production of type 1 INF by immature dendritic cells (DC) (Levy 2001). A loss of cells able to secrete INF has been associated with an increase in viral loads and a correlation between loss of INF secreting cells in individuals that proceed to AIDS compared to LTNP has been observed (Soumelis, Scott et al. 2001). The INF increases the expression of MHC-I and B7 on professional APC and is important for priming the humoral and cellular response (reviewed in (Levy 2001)).

During a primary HIV infection (PHI) the host is able to generate an HIV specific humoral response. The detection of antibodies provided the first diagnostic tests for HIV infection. Unfortunately the antibodies generated during the PHI have little ability to control the virus. As mentioned in the HIV pathogen chapter, the envelope proteins SU

and TM interact in a non-covalent manner to form an oligomer, most likely a trimer, which acts as the binding complex for HIV (Lu, Blacklow et al. 1995). The non-covalent interaction fails to hold all the envelope proteins in this trimeric form and thus HIV SU proteins can disassociate from the virion. It is believed that the antibodies generated during the primary infection are directed against free monomeric SU proteins and TM proteins left anchored to the cell or viral membrane. When the SU and TM proteins are in their trimer the antibody-binding sites are not exposed and thus the antibodies are unable to neutralize the virus (Figure 4.1) ((Moore, Cao et al. 1995) and reviewed in (Burton 1997)).

Another problem facing the development of neutralizing antibodies is that the SU protein is highly glycosylated. It has been reported that this glycoprotein is by weight 40-50% carbohydrates (reviewed in (Poignard, Saphire et al. 2001)). Since glycosylation of the SU protein occurs in the exogenous pathway of the host cell, similar to host cell proteins, the carbohydrates mask the HIV SU protein in a shield of self-antigens. This self-shield is believed to make it more difficult to develop neutralizing antibodies (Nab). Antibodies able to neutralize the virus take longer to appear, sometimes months or even years (reviewed in (Morris 2002)). Currently there are three broadly acting Nab targets that have been identified, two on the SU protein, b12 and 2G12, and one on the TM protein, 2F5. The antibodies that recognize these antigens are from the antibody class IgG. The b12 antibody-binding site is in the region that forms the CD4 binding site. The 2G12 antibody-binding site has been shown to be primarily composed of carbohydrates particularly mannose. The 2G12 is believed to interfere with the co-receptor binding. The 2F5 binding site is a linear amino acid sequence, ELDKWA. Since these neutralizing antibodies are not present during the PHI they are believed to participate in the control of SHI.

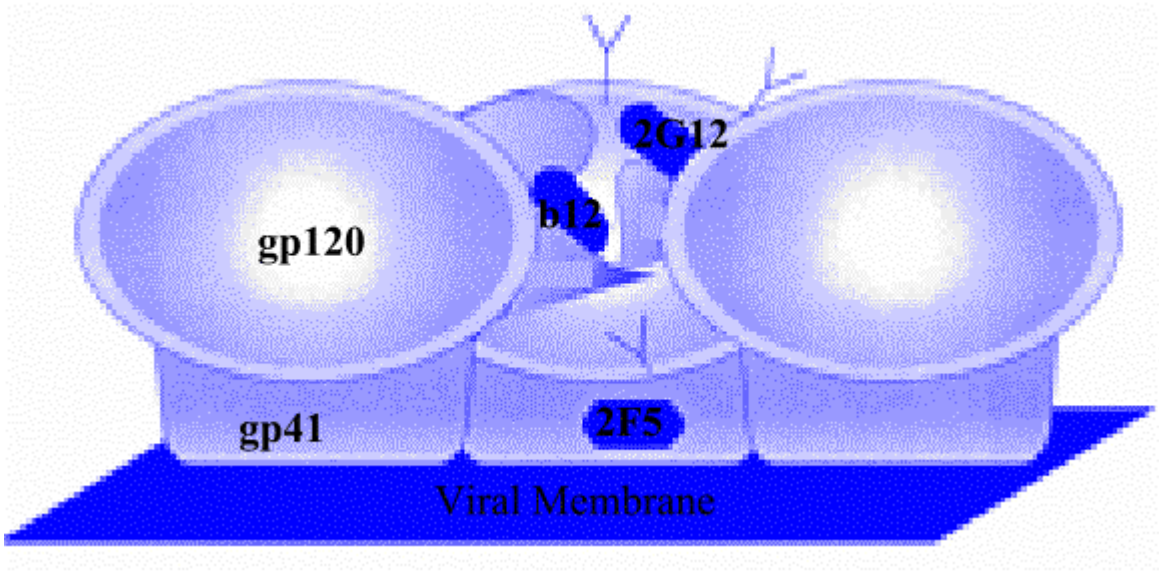


Figure 4.1 Schematic depiction of the HIV binding complex trimer.

The trimer is composed of three SU (gp120) and three TM (gp41) proteins. The three known neutralizing antibody sites are b12, 2G12, and 2F5. (Modified from (Burton 1997))

Since the antibody response is ineffective, it would presumably be the cellular response that causes the drastic down slope in the peak viral load. The graph below, Figure 4.2, was taken from Andrew McMichael's article in Nature 2001, but Dr. Wilson's group out of Sydney conducted the original work (Wilson, Ogg et al. 2000; McMichael and Rowland-Jones 2001). I have included this graph to demonstrate the host's ability to generate a strong CTL response against HIV during PHI. On the X axis are days after HIV infection and the Y-axis represents two variables, on the left is the % of CTL's from peripheral blood monocytes that recognize the HLA-B27 $Gag_{263\text{-}272}$ epitope as determined by MHC tetrameric studies and the right is the viral load based on

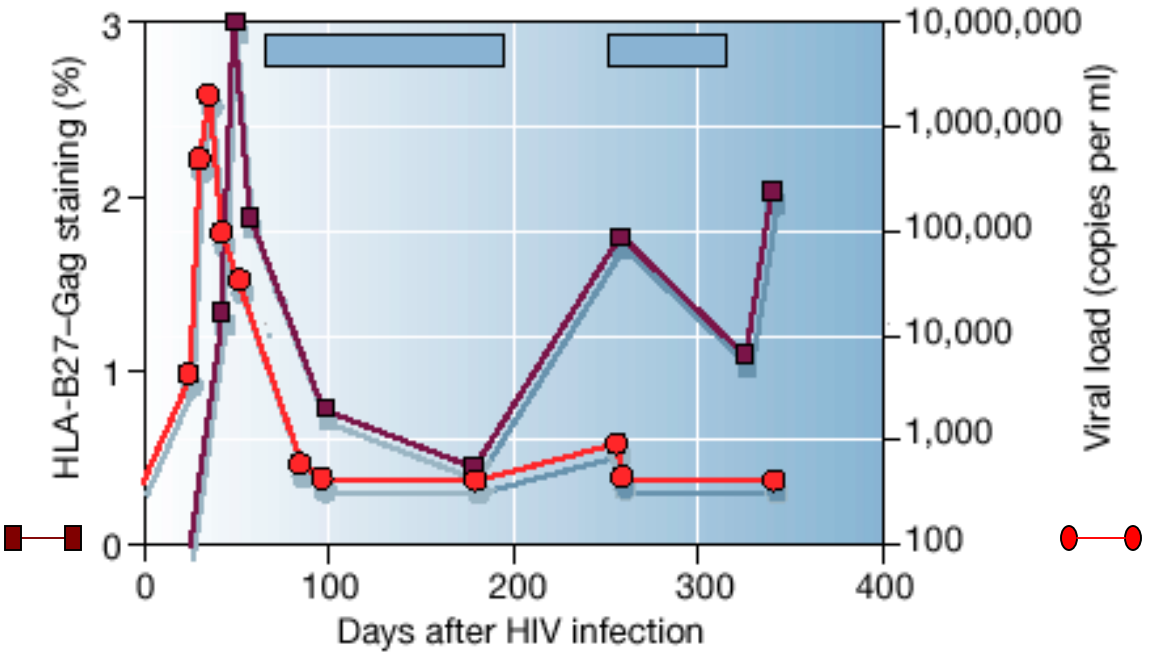


Figure 4.2 The CTL response compared to the viral load.

The CTL response as shown by HIV $Gag_{263-272}$ antigen specific tetramer staining compared to the viral load over time. The blue bars at the top indicate when HAART was employed. Taken from (McMichael and Rowland-Jones 2001) with the original work being conducted by (Wilson, Ogg et al. 2000).

the copies of RNA per ml. The blue bars at the top of the graph indicate treatment with HAART. At the start of the infections there is a sharp increase in the viral load (red line) peaking around week 5 at over 2 million copies/ml. But after the peak there is an equally sharp decrease in the viral load beginning prior to the start of HAART. This decrease appears to correspond with the appearance and peak of HIV specific CTLs (maroon line). These effector CTLs begin to show around the third week of infection and peak during the seventh week. Shortly after the viral load decreases, due to the lytic activity of the CTL there is a decrease in the HIV specific CTLs. The CTL count further decreases

when HAART is started in the 9th week. When HAART is interrupted from about the 200-250 day there is a minor increase in the viral load. More importantly, however, there is a sharp increase in the HIV specific CTLs, demonstrating a strong CTL memory response that is able to keep the viral replication in check. Thus, the memory CTL response is important during the SHI.

Over time the pressure exerted by the CTL response and later by the antibody response begins to waver as escape mutants start to appear. The development of these mutants can be traced back to two fundamental aspects about HIV, first, that HIV's RT protein does not possess proofreading capabilities and second that the primary receptor for HIV is the CD4 molecule. It is estimated that RT's failure to proofread causes 1 mutation in the HIV genome every cycle (reviewed in (Johnson and Desrosiers 2002)). This may not seem like much, but given that an infected individual produces HIV particles at a rate estimated between 10^7 and 10^8 virions per day, this would lead to enormous diversity (Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995). Furthermore, it has been shown that within an infected individual the DNA coding for the Env polyprotein can vary in sequence up to 10%. This diversity becomes a problem as the $CD4^+$ cell count decreases. Since HIV primarily infects the $CD4^+$ T_H-cells, as the host cell's CTL response destroys these cells, they slowly become depleted (Figure 1.2). Without the $CD4^+$ T_H-cells, naïve B-cells and T-cells fail to receive the co-stimulatory signals they need to properly differentiate and proliferate (Champagne, Ogg et al. 2001).

Not only does HIV evade the immune response by generating mutations in potentially antigenic areas, but they also have evolved a way to evade detection. As mentioned in the HIV pathogen chapter, HIV's Nef protein down-regulates the surface expression of MHC-I and CD4 molecules and up-regulates the expression of the FASL (Xu, Screaton et al. 1997; Collins, Chen et al. 1998; Fackler and Baur 2002). These

functions both hide the HIV infected cell from the CTL response and destroy CTLs that recognize HIV infected cells. Most interesting is that Nef specifically down-regulates classical MHC-I molecules, HLA-A and HLA-B, but not HLA-C and non-classical HLA-E. Not discussed in the immune system review was the activation of natural killer cells (NK), which have been shown to lyse cells not expressing HLA-C and HLA-E, (reviewed in (Biassoni, Cantoni et al. 2000)). Thus, by not down-regulating HLA-C and HLA-E the HIV infected cell avoids the effect of the NK cells.

The Tat protein also participates in the evasion process by down-regulating the MHC-II molecule (Kanazawa, Okamoto et al. 2000) Down-regulation of MHC-II molecules is not a concern in T_H-cells but presents a problem for macrophages. The macrophage, one of the antigen presenting cells, expresses low levels of CD4 and is therefore susceptible to HIV infection. Down-regulation of the MHC-II molecule should result in a decrease in antigen presentation to T_H-cells.

Perhaps HIV's most effective way of avoid the immune system is its ability to infect resting memory T_H-cells. After HIV has inserted its proviral DNA into the host genome it remains silent until the cell becomes active. During this silent phase, HIV does not produce proteins and thus remains completely undetectable. The only difference between an infected memory cell and an uninfected memory cell is the addition of 10 Kd to the total genomic mass, due to the proviral DNA. This makes it a very hard target for anti-viral drugs. Resting memory T-cells in uninfected persons have a mean survival of 5 months but HIV infected memory T-cells are believed to have a half-life of 44 months (reviewed in (Blankson, Persaud et al. 2002)). When these cells do become active it is likely that they will be lysed but not before infecting a new set of resting T-cells, thus creating a never-ending cycle.

PROJECT APPROACH

Since there is an obvious need for a vaccine against HIV that can prime the cellular arm of the immune system, our lab has looked at various ways to accomplish this goal. (Pollack, Zhan et al. 1997; Buseyne, Chaix et al. 1998; Riviere and Buseyne 1998; Rowland-Jones, Dong et al. 1999; Wasik, Wierzbicki et al. 2000; Buseyne, Le Gall et al. 2001; Kaul, Rowland-Jones et al. 2001; Rowland-Jones, Pinheiro et al. 2001) One of the more promising methods we have developed involves the tethering of a viral epitope to the N-terminus of the β_2-microglobulin (β_2m) via a small flexible linker. Recall that β_2m is the highly conserved structural protein in the MHC-I complex and that the MHC-I complex has the task of presenting epitopes to the cytotoxic T lymphocytes. Upon looking at the structure of the MHC-I complex, Figure 3.9, it became apparent that the C-terminus of the viral epitope was in line with the N-terminus of β_2m. From this observation it was postulated that by linking the two, one could present viral specific epitopes to the cellular immune system in the context of the MHC-I complex (Figure 5.1).

A detailed description of the rationale behind the idea can be found in Dr. James Caras's dissertation (Caras 1999). Briefly, it had been known for years that exogenous peptides could exchange with native peptides associated with the MHC-I molecule (Chen and Parham 1989). Also it had been shown that mice previously vaccinated with synthetic exogenous viral specific peptides could protect the mice from a lethal viral challenge (Kast, Roux et al. 1991). The peptide used was defined as an immunodominant CTL epitope from the Sendai virus nucleocapsid protein ($NP_{324-332}$). The use of peptides

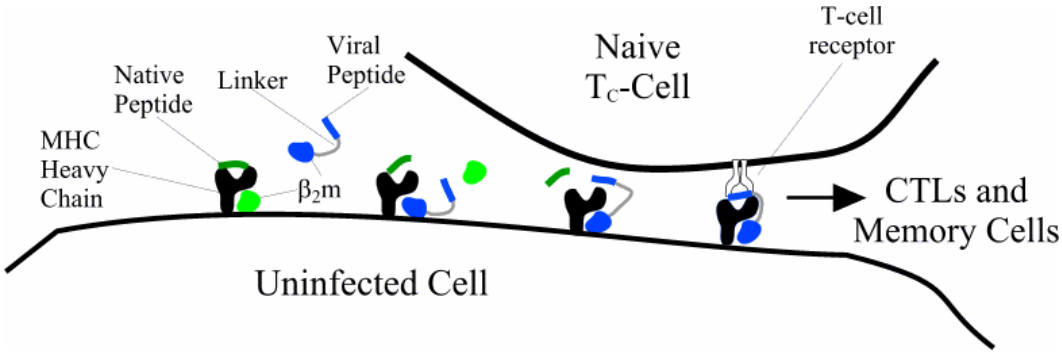


Figure 5.1 Depiction of the presumed β_2m-viral epitope exchange processes.

Presumably the β_2m linked to a viral peptide could exchange with native peptide and β_2m. Once exchange occurs the viral peptide present in the context of the MHC-I complex would stimulate CTLs to proliferate.

as potential vaccines had also been examined to protect sheep from bovine leukemia virus, a retrovirus (Hislop, Good et al. 1998). Four of the eight sheep immunized showed the desired CTL response even in the absence of a detectable antibody or $CD4^+$ T_H-cell response. Presence of proviral DNA was used as a measure of protection; sheep showing the desired CTL response were void of proviral DNA, whereas both sheep that displayed no CTL response and unvaccinated sheep showed detectable amounts. It should be noted, however, that peptides are very rapidly cleared from the bloodstream, greatly limiting their potential as effective vaccines.

There was also literature available showing that β_2m in serum readily exchanged with β_2m bound to the side of the MHC-I molecule (Bernabeu, van de Rijn et al. 1984). "The separate evidence demonstrating both peptide and β_2m exchange, as well as the

perceived spatially proximal and complementary ends of the displayed peptide and bound β_2m, led to the postulation of a novel vaccine comprised of a viral CTL epitope linked by a short peptide linker to β_2m. This construct could therefore present a viral antigen to cytotoxic T-lymphocytes by exchanging with native β_2m and peptide bound to cell-surface MHC I, thereby directly priming the cytotoxic immune response for subsequent protection against the virus" (Caras 1999).

The model system chosen to test the vaccine efficacy was the protection of mice from a lethal Sendai virus challenge. The Sendai virus is an enveloped negative-stranded RNA virus that belongs to the Paramyyxoviridae family and *Paramyxovirus* genus. Other members of this genus included the human parainfluenza virus type 1 and type 3 as well as the bovine parainfluenza virus type 3. The approximate 15,500 nucleotide genomic RNA of the Sendai virus encodes for 6 unique genes: nucleocapsid protein (NP), phosphorylated nucleocapsid-associated protein (P), large nucleocapsid-associated protein (L), nonglycosylated matrix protein (M), hemagglutinin-neuramindase glycoprotein (HN), and the fusion glycoprotein (F). In addition to these 6 genes, 6 other variations of the P protein exist and are referred to as C, C′, Y1, Y2, V, and W. (Lamb 1996) The first four variations C, C′, Y1 and Y2 are generated when alternative translational start codons slightly up stream or down stream of the P protein start site are utilized. The use of these start codons results in mRNA in the +1 open reading frame in relationship to the P protein mRNA. The remaining two variations of the P protein come about when one or two additional G nucleotides are inserted due to as a result of the RNA polymerase "stuttering" in a short repeat of C nucleotides. These additional P gene products can be found in an infected cell but not in the Sendai virion. (Lamb 1996)

The Sendai virion is spherical in shape with a diameter of approximately 150 to 350 nm. The virion consists of two sections, the other envelope and the helical

nucleocapsid. Separating the two sections is a layer of the M protein. The other envelope is made up of the previous host's lipid bilayer, the HN protein and the F protein. HN and F are responsible of adhesion and cell entry. The helical core is composed of the negative stranded RNA genome and approximately 2600 copies of NP, 300 copies of P and 50 copies of L (Lamb 1996). After the virus has infected a cell the NP, P, and L proteins come together to former the RNA transcriptase, which is responsible for transcribing and replicating the negative stranded RNA.

Although the Sendai virus is a negative stranded RNA virus and not a retrovirus similar to HIV, there are some features of this virus that make it a good model to test out the vaccine. First like HIV, protection from the Sendai virus requires a strong CTL response. Second, a well-characterized immunodominant CTL epitope from the NP protein is known (Kast, Roux et al. 1991). Third, a lethal dose of Sendai virus could kill unprotected mice within 2-3 weeks. Finally, the virus is non-transferable to humans.

Dr. Caras performed the initial work towards this goal. He tethered the immunodominant CTL epitope ($NP_{324-332}$) from the Sendai virus nucleocapsid protein (Kast, Roux et al. 1991) to the N-terminus of β_2m via a flexible 21 amino acid (AA) linker using extension PCR. He then cloned and expressed the construct in *E. coli*. After purifying the protein, he demonstrated that the recombinant protein was able to exchange with native β_2m on the surface of EL-4 cells *in vitro* (Figure 5.2). He showed that the construct was able to stabilize the MHC-I complex on the surface of RMA-S cells (Figure 5.3). Finally, Caras showed that the construct could stimulate viral specific T-cell hybridomas (Figure 5.4).

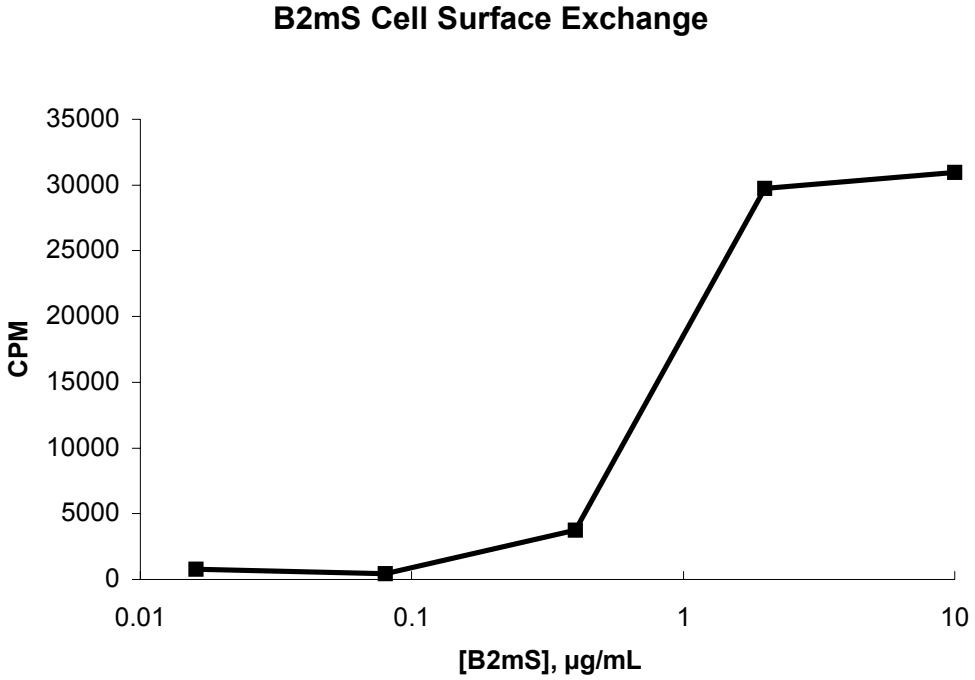


Figure 5.2 Cell surface binding/exchange of β_2mS.

Taken from Dr. Caras' Dissertation (Figure 33). RMA (H-2Kb) cells were incubated with 125I-radiolabeled β2mL21SE (β2mS) in a serum free media. Cells were washed and the radioactivity was measured in a gamma counter. A half saturating concentration of ~1 µg/ml and a maximum binding concentration of ~2 µg/ml was observed.

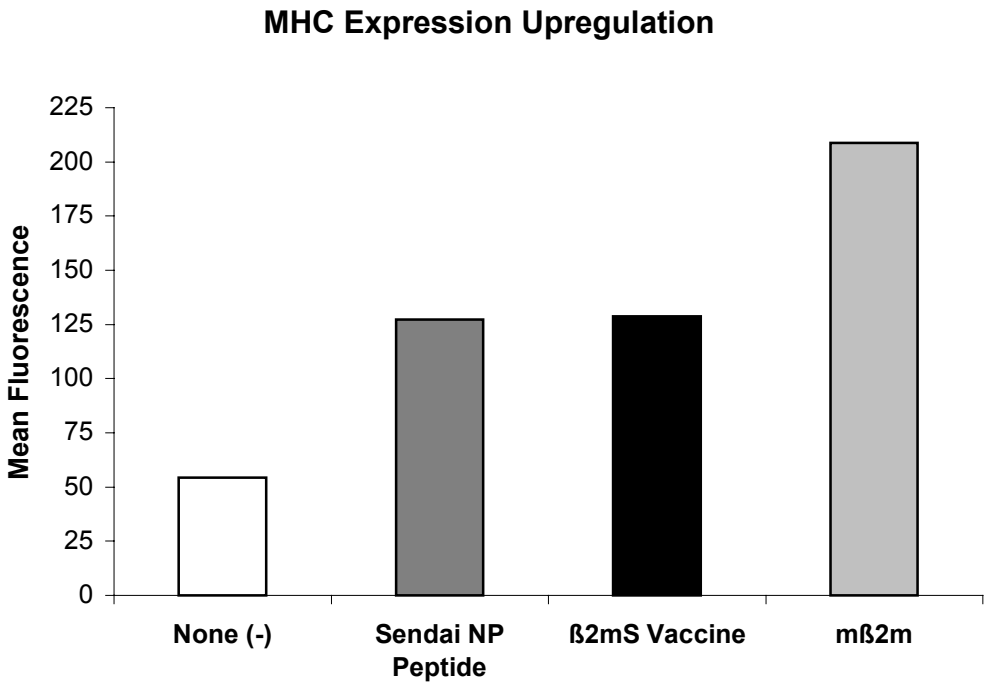


Figure 5.3 Upregulation of MHC Expression.

Taken from Dr. Caras' Dissertation. RMA-S cell were incubated with 100 μM β_2mL21SE (β2mS), the Sendai NP peptide, or mouse β_2m. Cells were then labeled with anti-mouse MHC heavy chain and the mean fluorescence was determined by flow cytometry.

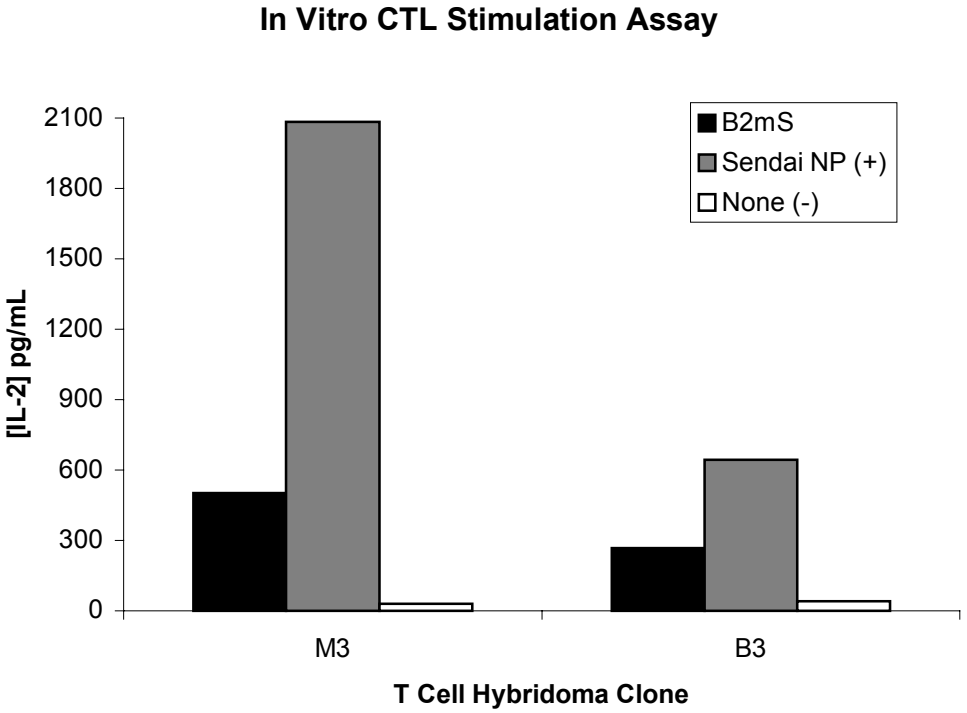


Figure 5.4 T-cell hybridoma stimulation assay.

Taken from Dr. Caras' dissertation (Caras 1999). EL-4 cells were first incubated with equal molar amounts of either β_2mL21SE (B2mS) or the Sendai NP peptide to allow for the exchange with native β2m and peptide or just the peptide alone. These target cells were then incubated with T-cell hybridomas (M3 and B3) reactive to the Sendai epitope. The ability to stimulate the hybridomas was determined by monitoring the release of IL-2 in the supernatant by a standard sandwich ELISA.

The overall conclusion from Caras' work was that the β_2m- 21 AA linker- Sendai epitope fusion protein could exchange with native β_2m and peptide and trigger the release of IL-2 from viral specific T-cell hybridomas, *in vitro.* Since the vaccine was less efficient at stimulating the hybridomas than the peptide alone it was speculated that the linker might be interfering with the TCR binding to the MHC-I molecule. Caras chose the length of the linker by adding a few amino acids to the minimum length that was needed to fuse the C-terminus of Sendai epitope to the N-terminus of β_2m, based on molecular modeling he performed using the published crystal structure data (Fremont, Mutsamura et al. 1992).

At about the same time that Caras was performing the initial work on the β_2m peptide vaccine, Dr. Barber's lab at the University of Toronto published an article in 1998 in the Journal of Immunology that showed that both cells pulsed with a recombinant fusion protein of human β_2m tethered via 12 AA to a H-2D^b-restricted immunodominant epitope from influenza virus NP and cells transfected with a plasmid encoding β_2m linked via 8 AA to a H-2K^d-restricted influenza NP epitope could function as target cells in a chromium release assay with splenocytes from influenza infected mice (Uger and Barber 1998). However, neither Caras nor Uger examined the effect of linker sizes on TCR recognition. But more importantly neither group assayed the ability of the β_2m fusion vaccine to trigger an immune response *in vivo* or its ability to provide protection from a viral challenge. Both the effect of linker size and the vaccine's protective qualities as either a recombinant protein expressed in *E. coli* or as part of a mammalian expression vector are presented in this work.

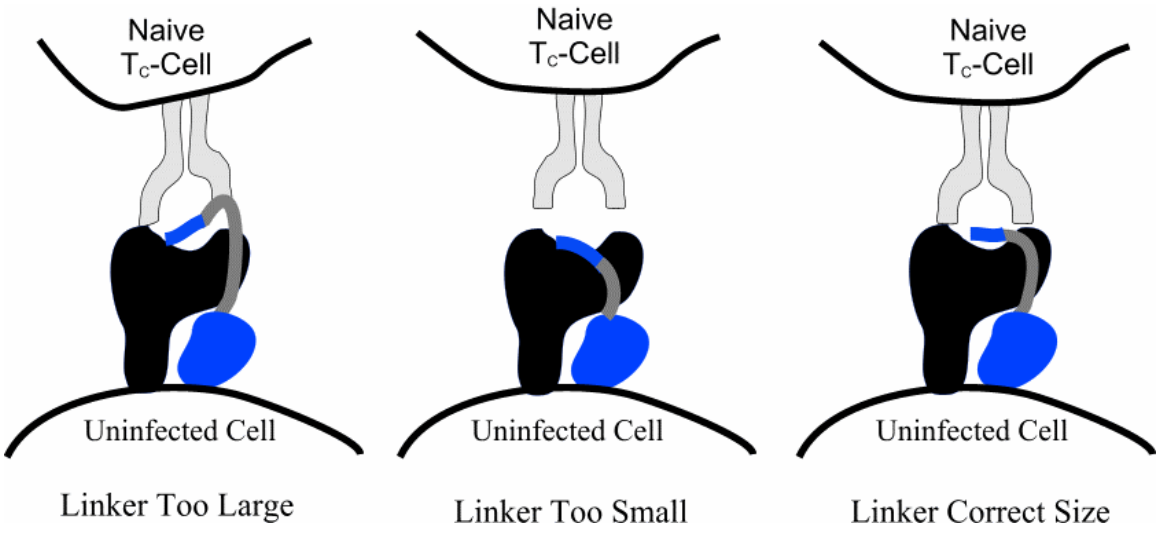


Figure 5.5 The presumed effect linker size has on T-cell receptor recognition of the MHC-I molecule.

One can imagine that the linker could interfere with the TCR's interaction with the MHC-I molecule in the following manner (Figure 5.5). If the linker was too large one might expect that a bulge could develop near the C-terminus of the Sendai epitope thus causing interference with viral specific T-cell hybridoma recognition. If the linker was too small one might expect that the Sendai epitope would be unable to set appropriately in the MHC heavy chain groove. Given that Caras chose a length that was above his predicted length, I was charged with engineering two additional β_2m Sendai epitope fusion proteins: one with a 15 AA linker and the other with a 10 AA linker. Once the best linker size was determined it would be tested *in vivo* to see if it could elicit a viral specific CTL immune response and protect against a lethal viral challenge.

In addition to the potential of peptide vaccines, much work has been published on the potential of naked DNA to stimulate the cytotoxic T-lymphocyte portion of the cellular immune response in both animals and humans. Wolff et al., 1990, demonstrated that naked DNA encoding standard marker genes such as beta-galactoside or luciferase could produce detectable protein up to two months after injection into muscles of mice (Wolff, Malone et al. 1990). Then in 1993, Ulmer, et al, showed that mice vaccinated intramuscularly with a plasmid expressing the influenza A nucleocapsid protein was able to protect mice against a lethal challenge of influenza A (Ulmer, Donnelly et al. 1993). Also that same year, Robinson, et al. showed that a plasmid expressing the influenza haemagglutinin protein was able to protect 50% of vaccinated chickens from a lethal challenge of influenza (Robinson, Hunt et al. 1993). Since these initial studies many other labs have shown the protective potential of DNA vaccines. (Barouch, Santra et al. 2000; Johnson, Conway et al. 2000; Shata and Hone 2001; van Rooij, Glansbeek et al. 2002)

A proposed mechanism by Gregersen as to how the DNA vaccine could elicit a CTL response is depicted in Figure 5.6 (Gregersen 2001). In this model, injected naked DNA is taken up by the muscle cell, which then expresses the protein encoded by the plasmid. At this point the translated protein could get tagged for degradation, processed via the cytosolic pathway, and be presented in the MHC-I complex to $CD8^+$ T-cells. Alternatively the protein may be secreted or released in to the extracellular fluids. There professional antigen presenting cells, such as macrophages or dendritic cells, would be able to endocytose the protein and present epitopes to $CD4^+$ T-helper cells via the endocytic pathway. Presentation to the T-helper cells would cause the secretion of cytokines that could benefit the cytotoxic T-cell response or the antibody response.

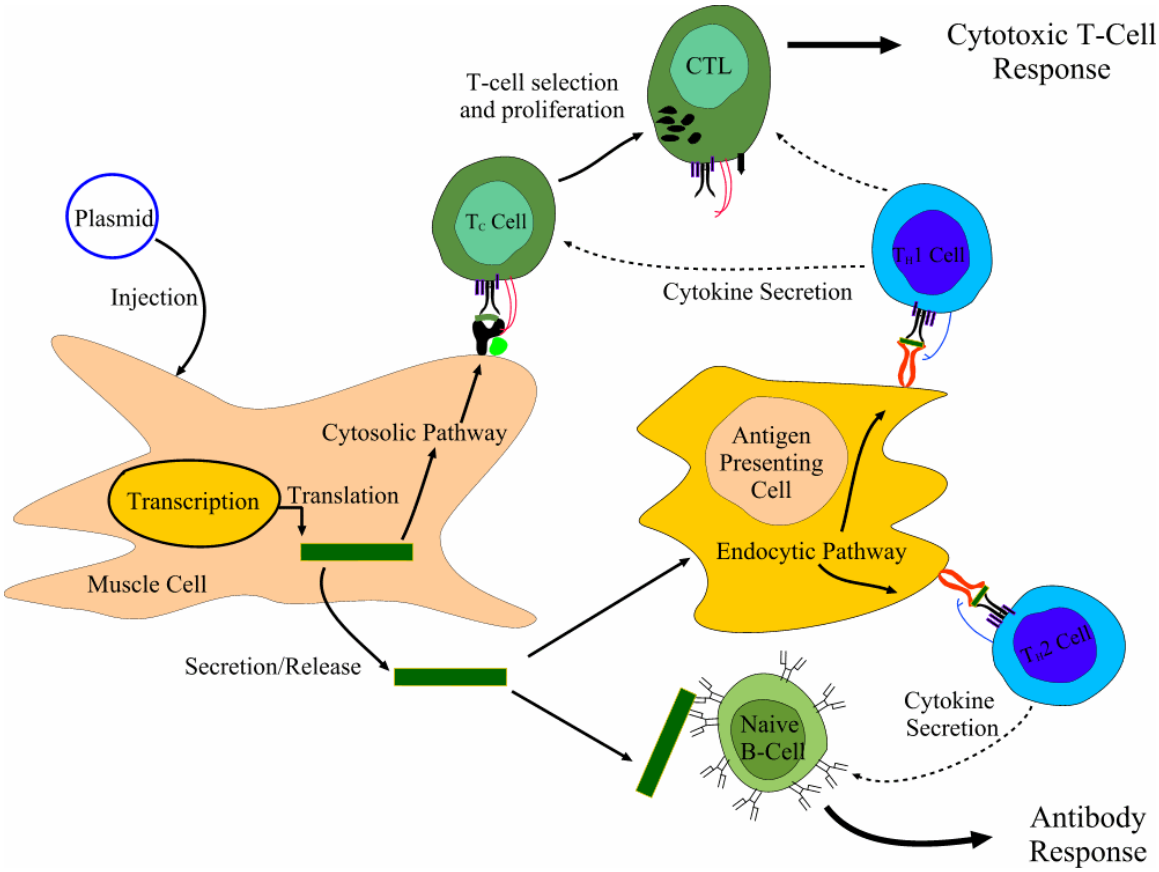


Figure 5.6 A proposed mechanism showing how a DNA vaccine could elicit an immune response.

Modified from Fig. 1 Gregersen, 2001 (Gregersen 2001) and described in text.

Given the promising results from DNA vaccine studies of others, it was believed that the β_2m-viral fusion construct with minimal modifications could be ideal as a DNA vaccine. The primary modification to the original design of the plasmid construct used for production of the tripartite fusion peptide vaccine in *E. coli* was adding a eukaryotic signal peptide that would direct the translation of the fusion protein directly into the endoplasmic reticulum of recipient cells. This was done because degradation in the cytosol was thought to be unwanted and unnecessary for association with the MHC heavy chain. Since the optimal linker size had not been determined, I was given the opportunity to develop the DNA vaccine with the β_2m-viral epitope-signal peptide fusion construct utilizing three different linker sizes: 10 AA, 15 AA, and 21 AA. Again, once the best linker size had been determined *in vitro*, the vaccine would be assayed *in vivo* for its ability to generate a viral specific CTL response and its capacity to protect against a lethal viral challenge.

RESULTS

β_2m-Sendai Epitope Vaccine as a Recombinant Protein

PEPTIDE VACCINE CONSTRUCT DESIGN AND CLONING

The design of the β_2-Microglobulin-Sendai Epitope peptide vaccine involved the fusion of β_2-microglobulin (β_2m) to the immunodominant CTL epitope of the Sendai virus nucleocapsid protein (NP). The Sendai epitope was taken from NP amino acids 324-332 (FAPGNYPAL), which had been reported to be the most immunogenic region of the virus (Kast, Roux et al. 1991). In order for the Sendai epitope to load correctly into the MHC heavy chain, a spacer linking the N-terminal of β_2m to the C-terminal of the Sendai virus NP epitope was required. The optimal length of the linker was unknown, so three linker sizes were chosen: 10, 15 and 21 amino acids (AA). Linkers were composed of glycines and serines to provide flexibility and low immunogenicity. For ease of purification an N-terminal poly-histidine tag was added. An enterokinase site was also included to allow for removal of the poly-histidine tag and to ensure that the N-terminal of the fusion protein was the first AA residue of the Sendai epitope.

Cloning of β_2-microglobulin-Sendai epitope vaccine with a 21 AA linker and a poly-histidine tag (β_2mL21SEHT)

The design and cloning of the β_2-microglobulin-Sendai vaccine with a 21 AA linker (β_2mL21SEHT) is described in detail in Dr. Jamie Caras' Dissertation, 1999; therefore, it will not be discussed in detail in this writing (Caras 1999). The end result was a pET-21a plasmid (Novagen) with an insert coding for β_2m-L21 amino acid linker-Sendai epitope ($NP_{324-332}$) – poly-histidine tag. In Dr. Caras's dissertation β_2mL21SEHT

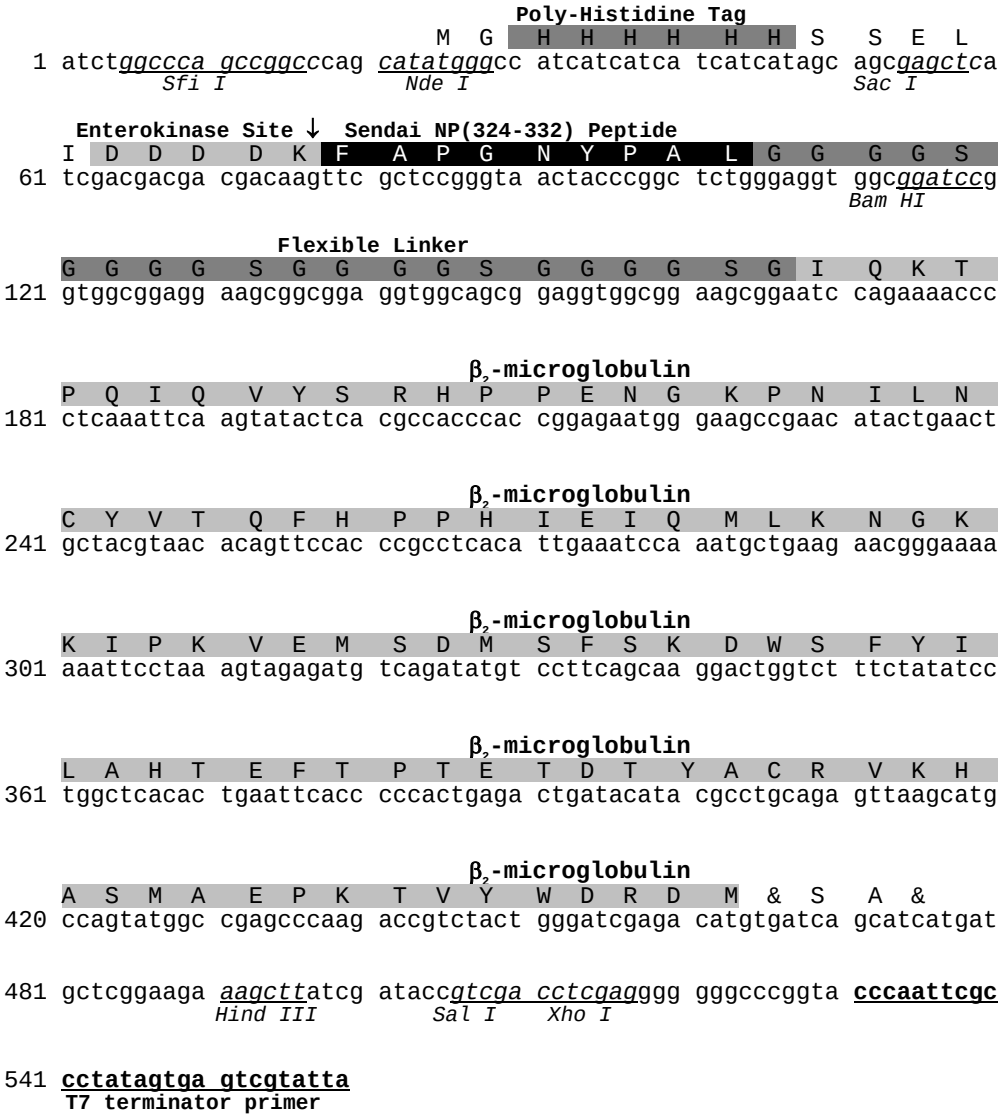


Figure 6.1 The sequence of β_2mL21SEHT.

Taken from Dr. Caras's dissertation (1999) 'Figure 26: Nucleic acid and protein sequence of β_2mS construct.'

is referred to as β2mS; however, throughout this presentation it will have the abbreviation of β_2mL21SEHT to distinguish it from both the peptide vaccine constructs with other linker sizes and the DNA vaccine constructs. Figure 6.1 gives the construct DNA sequence and protein sequence; and highlights the various segments of the construct. Restriction digestion sites are underlined and an arrow identifies the location of the enterokinase cut. The flexibility to exchange epitopes was integrated into the construct by surrounding the region coding for the epitope with a Sac I and a BamH I endonuclease site.

Cloning of β_2-microglobulin-Sendai epitope vaccine with a 10 or 15 AA linker and a poly-histidine tag (β_2mL10SEHT, β_2mL15SEHT)

Dr. Caras obtained a pBluescript-SK vector coding for the murine β_2-microglobulin (pSK-β_2m) from Dr. Stanley Nathenson at the Albert Einstein College of Medicine of Yeshiva University. (Sakita, örig et al. 1996). This was also used in the current work. For the generation of β_2-microglobulin-Sendai epitope vaccine with a 10 or 15 amino acid linker and a poly-histidine tag, β_2mL10SEHT and β_2mL15SEHT, respectively, this pSK-β_2m acted as the initial template in a two-step extension PCR. For each PCR reaction, the T7 terminator primer was used as the downstream primer. The first extension primer overlapped 18 nucleotides on the N-terminal of β_2m in the pSK-β_2m plasmid and added the linker of 10 or 15 AA. The second primer annealed to the linker and added the Sendai epitope and enterokinase site (Figure 6.2). A byproduct of the PCR reactions was the addition of adenosine nucleotides to the 3′ ends of the amplified product. This byproduct allowed for direct ligation of the purified PCR product 2 into the pCRT7/NT TOPO vector (Invitrogen). The vector encodes for its own enterokinase site and N-terminal poly-histidine tag. However, the vector's enterokinase

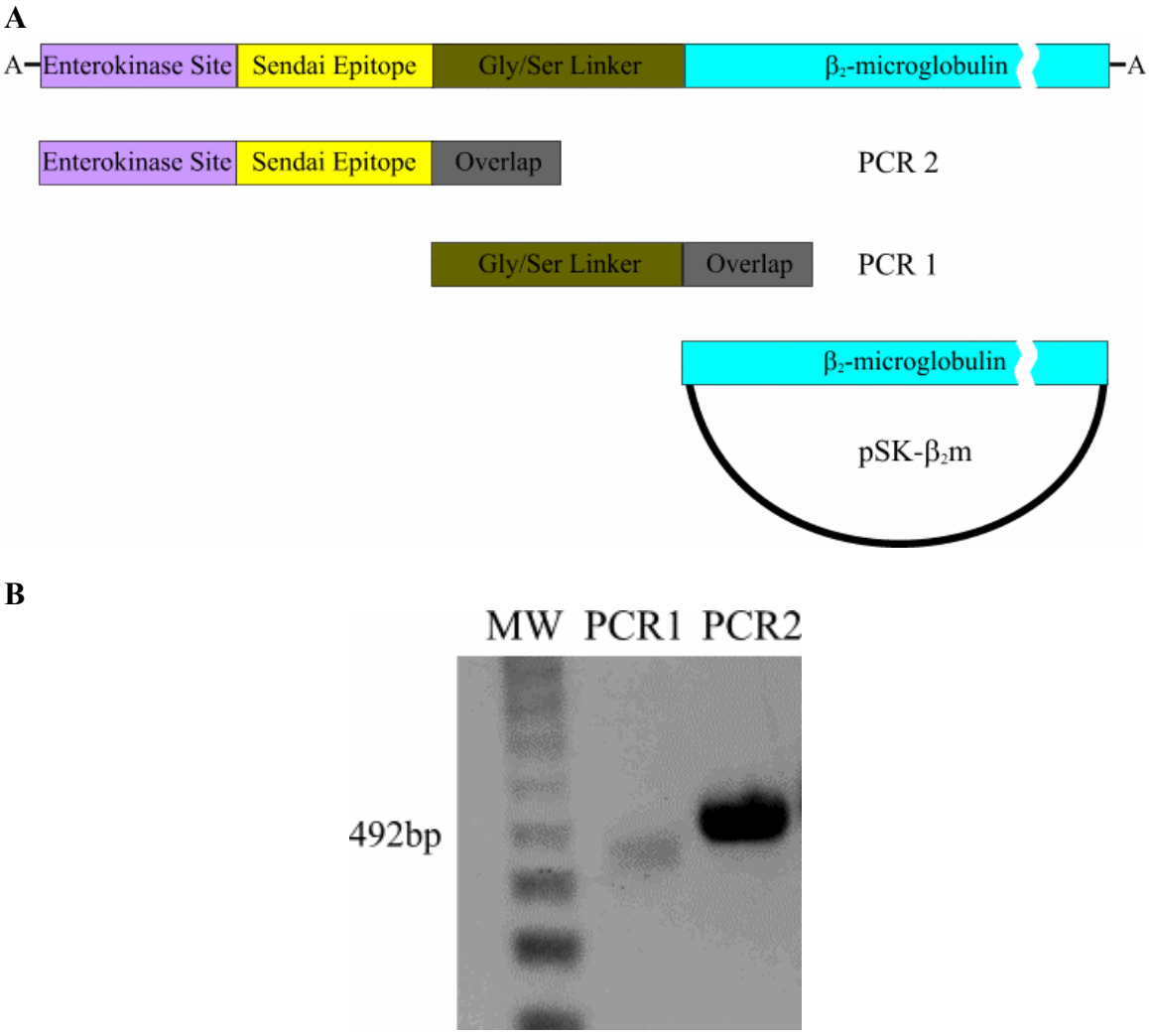


Figure 6.2 Peptide vaccine construct design.

A) Schematic depiction of the extension PCR used to constructed the β_2mL10SEHT and β_2mL15SEHT peptide vaccines. The first extension PCR adds the linker to the N-terminal of β_2m; the second PCR adds the Sendai epitope and enterokinase site. B) A color-inverted digital image of a 1% agarose gel showing the increase in β_2mL15SEHT construct size from PCR1 to PCR2. The downstream primer and upstream primers were at a final concentration of 0.3µM. The PCR reaction was set at 94.0°C for 5 min, then cycled for 30 times from 94.0°C for 45s -> 55.0°c for 45s -> 72.0°C for 60s. The template for the first reaction was the pSK-β_2m plasmid, while the templates for the second PCR reactions was the gel-purified product from the previous PCRs.

site does not cut at the N-terminal of the Sendai epitope, which was why an additional enterokinase site was added during PCR 2. The plasmids were transformed into and cloned in TOP10F' cells (Invitrogen). The cells were allowed to recover in (SOC) before being plated on LB/Agar plates with ampicillin. Using a PCR reaction, colonies were screened for the inserted vector (Figure 6.3). The plasmids were isolated from colonies showing the insert and then purified before being sequenced at the University of Texas Sequencing Center. Figures 6.4 and 6.5 give the construct DNA and protein sequences for β_2mL10SEHT and β_2mL15SEHT, respectively. Highlighted are the various segments of the construct, such as the poly-histidine tag, the enterokinase sites, the Sendai epitope, the linker and the murine β_2m. Restriction digestion sites are underlined and an arrow identifies the location of the enterokinase cut.

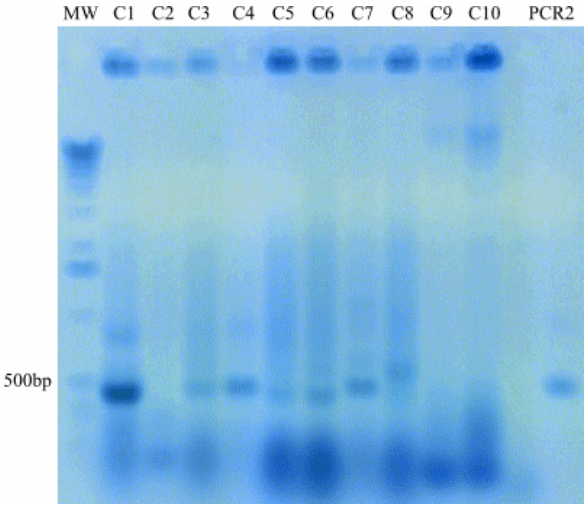


Figure 6.3 Colony PCR screen for β_2mL10SEHT.

A color-inverted digital image of a 1% agarose gel with the products of a colony PCR screen for β_2ML10SESP. β_2ML10SEHT was transformed into TOP10F' cells by heat shock at 42°C for 45 seconds. Cells were allowed to recover in SOC for 1 hour and then plated on LB plates containing 60 μg/ml ampicillin. With a toothpick individual colonies were chosen and then screen by PCR for the insert. C1-C10 correspond to the 10 colonies selected. PCR2 is the purified product from the second extension PCR for β_2ML10SEHT. MW is a DNA ladder.

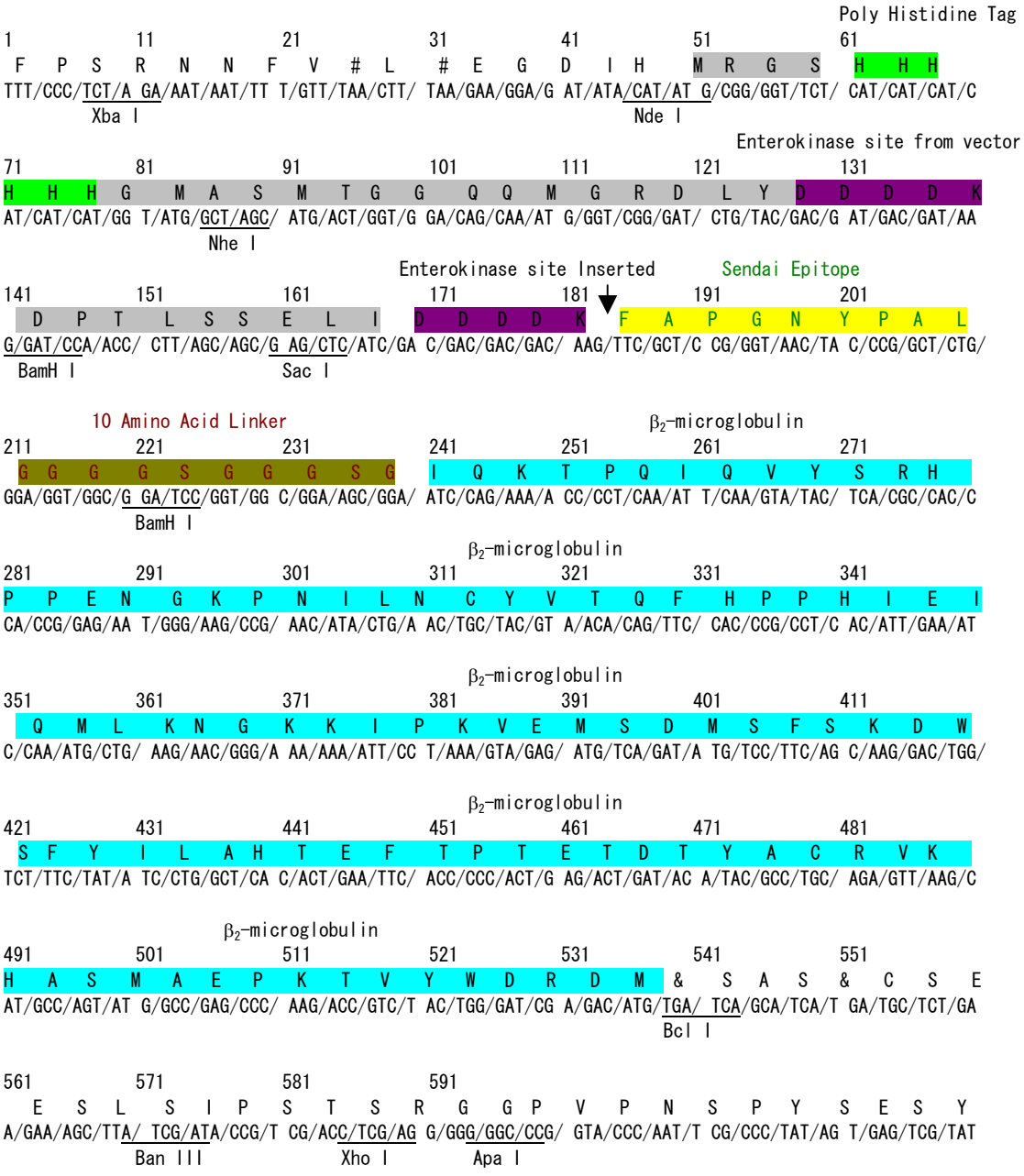


Figure 6.4 The sequence for pCRT7/NT β_2mL10SEHT.

TOP10F' cells were transformed by heat shock with pCRT7/NT β_2mL10SEHT and grown overnight in 5mls of LB with 60 μg/ml ampicillin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the reverse primer. The sequence is reported here in the 5'-3'direction.

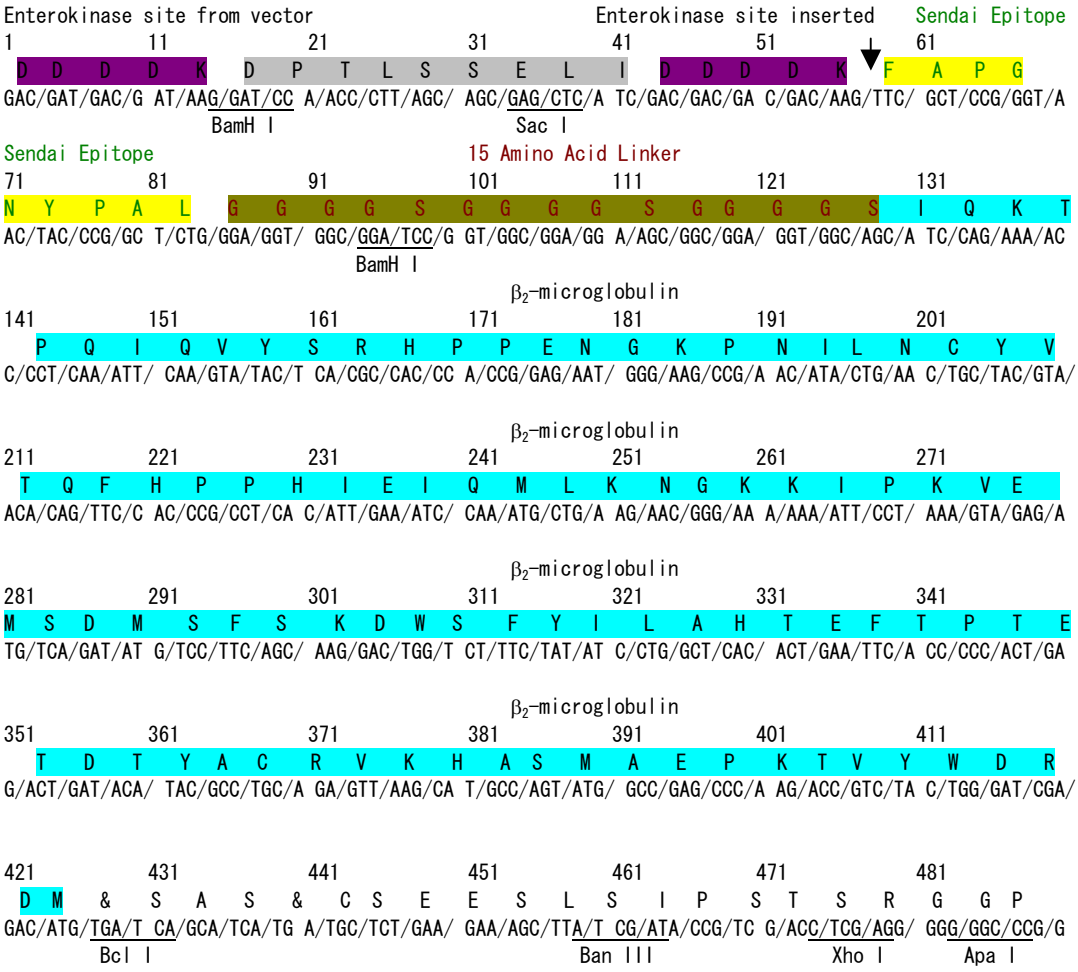


Figure 6.5 The sequence for pCRT7/NT β_2mL15SEHT.

TOP10F' cells were transformed by heat shock with pCRT7/NT β_2mL15SEHT and grown overnight in 5mls of LB with 60 μg/ml ampicillin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the forward primer. The primer bound just before the enterokinase site on the vector, thus for the poly-histidine tag sequence and start codon refer to the Invitrogen pCRT7/NT manual (Invitogen 2000). Also additional restriction sites can be found there.

Cloning of β_2-microglobulin-dummy epitope vaccine with a 15 AA linker and a poly-histidine tag (β_2mL15DMHT)

The original attempts to exchange the epitope coding sequence for Sendai $NP_{323-332}$ with the dummy epitope involved restriction-digesting the β_2mL21SEHT with Sac I and BamH I, then ligating in complementary oligos, so that when annealed it encoded the dummy epitope with Sac I and BamH I overhangs. This method, however, failed. Instead the construct for the dummy epitope was obtained by performing a one-step extension PCR. The template used was the pCRT7/NT β_2mL15SEHT and the downstream primer was the T7 terminator primer. The upstream primer was similar to the primer in the second PCR reaction above, except instead of encoding for the Sendai epitope it encoded the dummy epitope. This PCR product was gel purified and ligated into the pCRT7/NT TOPO plasmid. The plasmid was transformed into and cloned in TOP10F′ cells. Cells were plated on LB/Agar plates with ampicillin and were colony PCR screened for the insert. (Data not shown) The plasmid was isolated from a single colony and then purified before being sequenced at the University of Texas Sequencing core facility. Figure 6.6 gives the construct DNA sequence and protein sequence for β_2ML15DMHT. Highlighted are the various segments of the construct, such as the poly-histidine tag, the enterokinase sites, the Sendai epitope, the linker and the murine β_2m. Restriction digestion sites are underlined and an arrow identifies the location of the enterokinase cut.

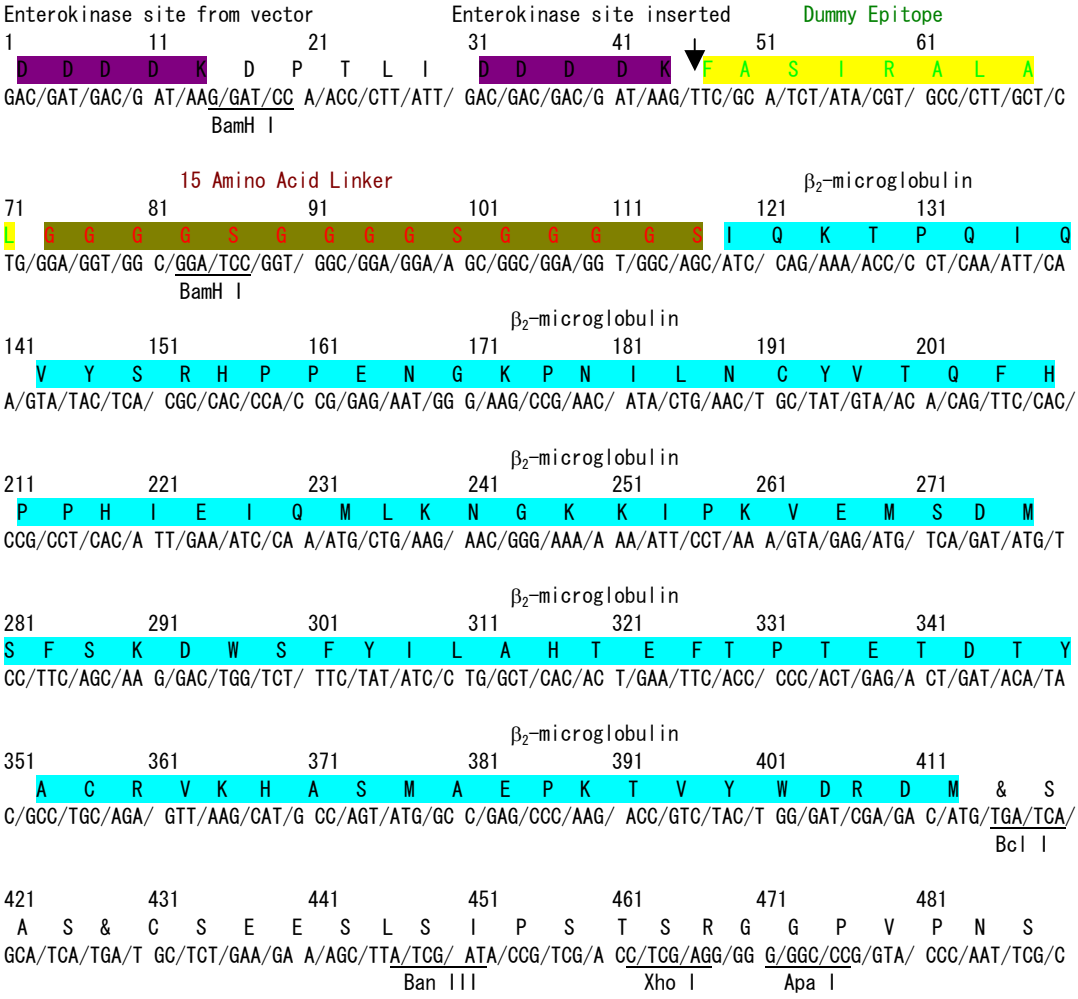


Figure 6.6 The sequence for pCRT7/NT β_2mL15DMHT.

TOP10F' cells were transformed by heat shock with pCRT7/NT β_2mL15DMHT and grown overnight in 5mls of LB with 60 μg/ml ampicillin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the forward primer. The primer bound just before the enterokinase site on the vector, thus for the poly-histidine tag sequence and start codon refer to the Invitrogen pCRT7/NT manual (Invitogen 2000). Additional restriction sites can also be found there.

EXPRESSION AND PURIFICATION

The verified plasmids were individually transformed into the BL21(DE3) vector and grown in LB with ampicillin. Expression was induced with IPTG for 2-4 hours before the cells were pelleted. The cell pellets were lysed with 8M urea and the cell debris was removed by centrifugation. The protein, containing the poly-histidine tag, was bound to a denatured Ni-NTA column and then eluted by lowering the pH. The protein was then refolded in a two-step dialysis in which the urea concentration was dropped from 8M to ~ 4M and then to ~ 30 mM. The poly-histidine tag was cut off the protein by digesting it with enterokinase (Novagen). Samples were then concentrated using a YM-3 Centricon or YM-3 Centripep (Millipore Corporation). The purification process (Appendix 1) was analyzed by SDS-PAGE (Figure 6.7). Uncut proteins were referred to as pβ_2mL10SEHT, pβ_2mL15SEHT, pβ_2mL21SEHT, and pβ_2mL15DMHT. Since the histidine tag was cut off, the final protein products were identified as pβ_2mL10SE, pβ_2mL15SE, pβ_2mL21SE, and pβ_2mL15DM.

MHC EXPRESSION UPREGULATION / STABILIZATION

An MHC expression upregulation experiment was performed to determine if the β_2m-Sendai epitope fusion proteins could stabilize the MHC complex on RMA-S cells (Zhou, Glas et al. 1993). The RMA-S cell line is void of TAP2, thus cytosolic peptide processed in the classical cytosolic pathway is unable to be shuttled across the ER membrane. This leads to MHC complexes presented on the outside of the cell, consisting of only the MHC heavy chain and β_2m. Without the peptide in the groove of the MHC heavy chain, these complexes are unstable and disassociate. The 'empty' MHC

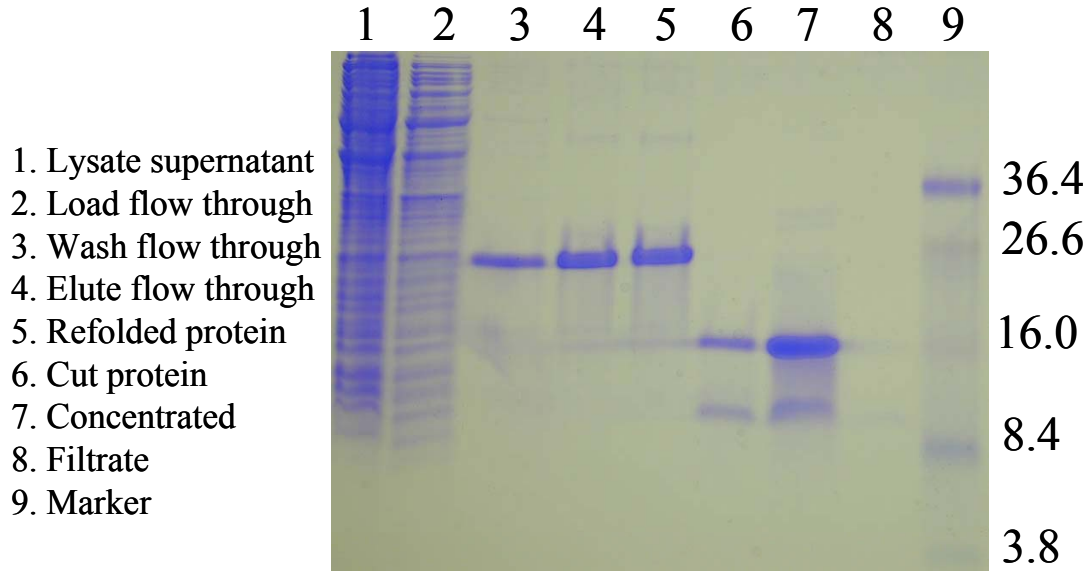


Figure 6.7 A 15% SDS-Page gel on pβ$_2$mL10SE vaccine purification, enterokinase digestion, and concentration.

A 10 ml starter culture of LB with 60 μg/ml ampicillin was inoculated with BL21(DE3) cells, which had been transformed with the pCRT7/NT TOPO vector coding for the β$_2$mL10SEHT protein, and grown overnight. The starter culture was used to inoculate 2 six-liter flasks containing 1400 ml of LB with 60 μg/ml ampicillin. The cells were grown to an OD_{600} between 0.7-0.8 Abs at which point they were induced with 1 mM IPTG. Protein expression was allowed to continue for 2-3 hrs before the cells were collected by centrifugation at 4500g for 15 min. The cell pellet was weighed and the cells lysed with 5 times (ml/g) of Lysis Buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris•Cl, pH=8.0). The cell debris was removed from the lysate by spinning at 27000g for 30 min. The supernatant (Lane 1) was loaded on to a Ni-NTA agarose column and the flow through was collected (Lane 2). The column was washed with Lysis Buffer (pH=6.3) and the flow through was collected (Lane 3). The protein was then eluted with Lysis Buffer (pH=4.5) (Lane 4). The pH of the collected protein was raised to 8 and then it was refolded in a two step dialysis first with buffer A (50 mM glycine, 10% (w/v) sucrose, 1 mM EDTA, 4 M urea, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, pH 9.6) and then with buffer B (60 mM ethanolamine, pH 9.6, 10% (w/v) sucrose, 1 mM EDTA, 0.1 mM reduced glutathione, 0.01 mM oxidized glutathione) (Lane 5). After refolding the poly-histidine tag was removed by cutting with 50 U of recombinant Enterokinase (Lane 6). The protein was concentrated using a YM-3 Centriprep spun at 3000g (Lane 7 = concentrated solution; Lane 8 = filtrate). The purification examined with a 15% SDS-PAGE gel with a molecular weight marker in lane 9.

complexes can be stabilized on the surface of the cells if they are pulsed with class I peptide. Thus, this system will allow easy determination if the Sendai peptide, at the N-terminal of the vaccine constructs, is properly loaded into the groove. This can be demonstrated by increased MHC-I (H-2K^b) staining as measured by flow cytometry.

RMA-S cells were incubated with 50 μM purified pβ_2mL10SE, pβ_2mL15SE, pβ_2mL21SE, or Sendai NP peptide: five samples each. The cells were then stained for MHC upregulation using a R-PE Anti-mouse H-2K^b antibody (BD Pharmingen), which can be detected in the FL2 channel of the flow cytometer. The negative controls for this experiment were RMA-S cells pulsed with nothing and stained for MHC-I and RMA-S cells pulsed with nothing and not stained with the MHC-I antibody. Samples were gated, after data collection, by forward scatter and side scatter measurements to isolate the live cells (Figure 6.8). Notice that there are very few cells in the live cell gate for Sendai peptide pulsed RMA-S cell. This concern will be addressed in the next experiment. Figure 6.9 presents representative stains demonstrating the shift in fluorescence indicative of the stabilization of the MHC-I molecule. Notice that in stained RMA-S cells the peak occurs close to 10^2, whereas in all fusion protein pulsed cells the peak shifts right to approximately 10^3. Unfortunately, there were not enough live cells in the peptide pulsed group to make sense of the data and thus it is not presented. Figure 6.10 depicts the average of the five samples and provides the confidence level, based on Student t-Test analysis, at which one can state that the values were distinct from one another.

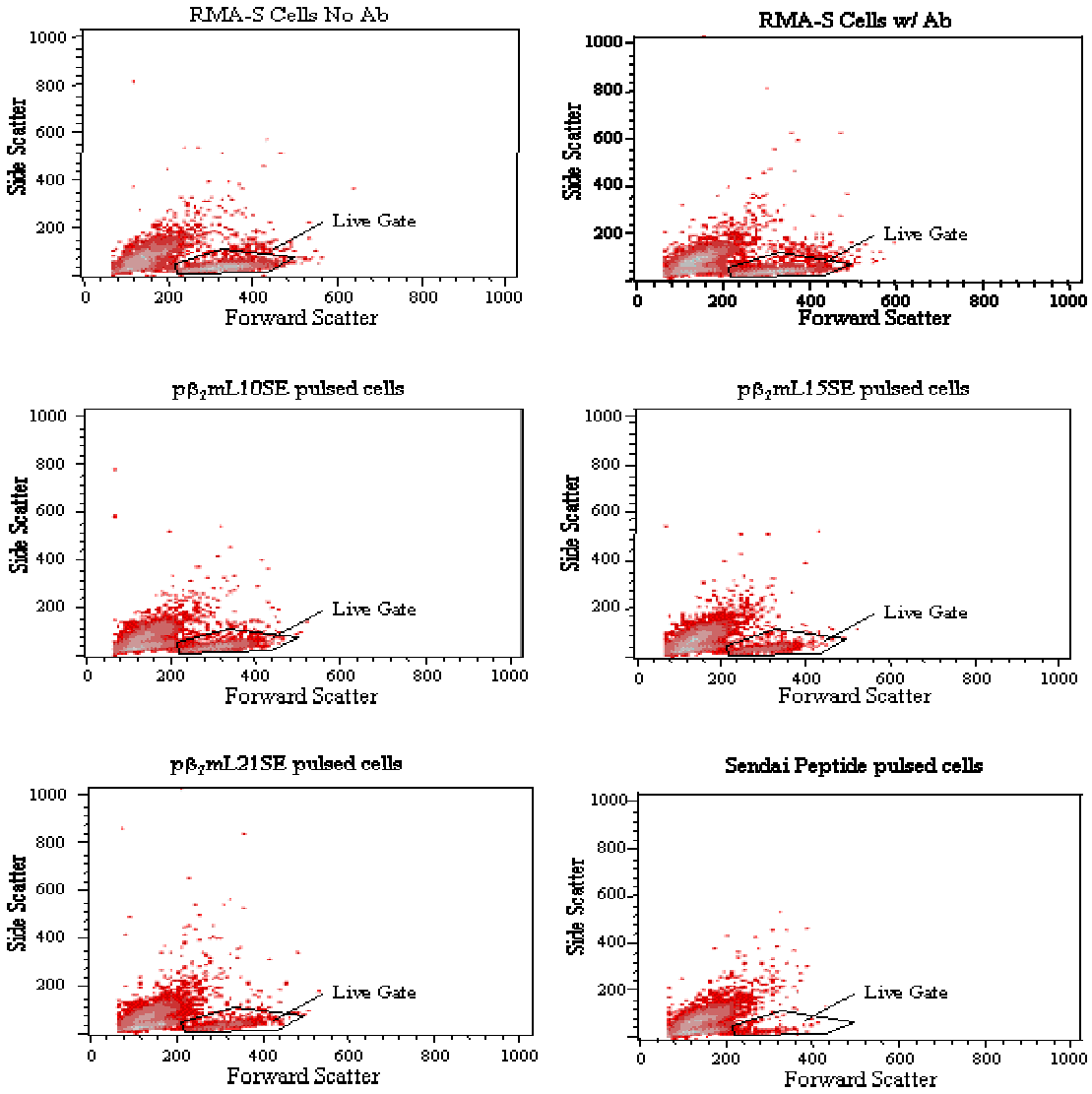


Figure 6.8 Live cell gate used for an MHC-I upregulation experiment.

RMA-S cells ($3.5x10^5$) were incubate with 50 µM of the given β_2m-Sendai epitope fusion protein, the Sendai peptide or nothing at all and grown at 30°C and 5%CO_2 for 1hr. The plate was sealed and the cells were then grown for 18-20hrs at 26°C. The cells were then placed at 37°C for 5% CO_2 for 2hr. The cells were pelleted and washed with wash buffer (PBS with 0.1% sodium azide). Cells were incubated with R-PE Anti-mouse H-2Kb (BD Pharmigen) at 2.0 µg/ml in wash buffer for 30 min on ice. Cells were washed twice and resuspended in 200 µl of wash buffer then stored on ice until analyzing the fluorescence via FACS.

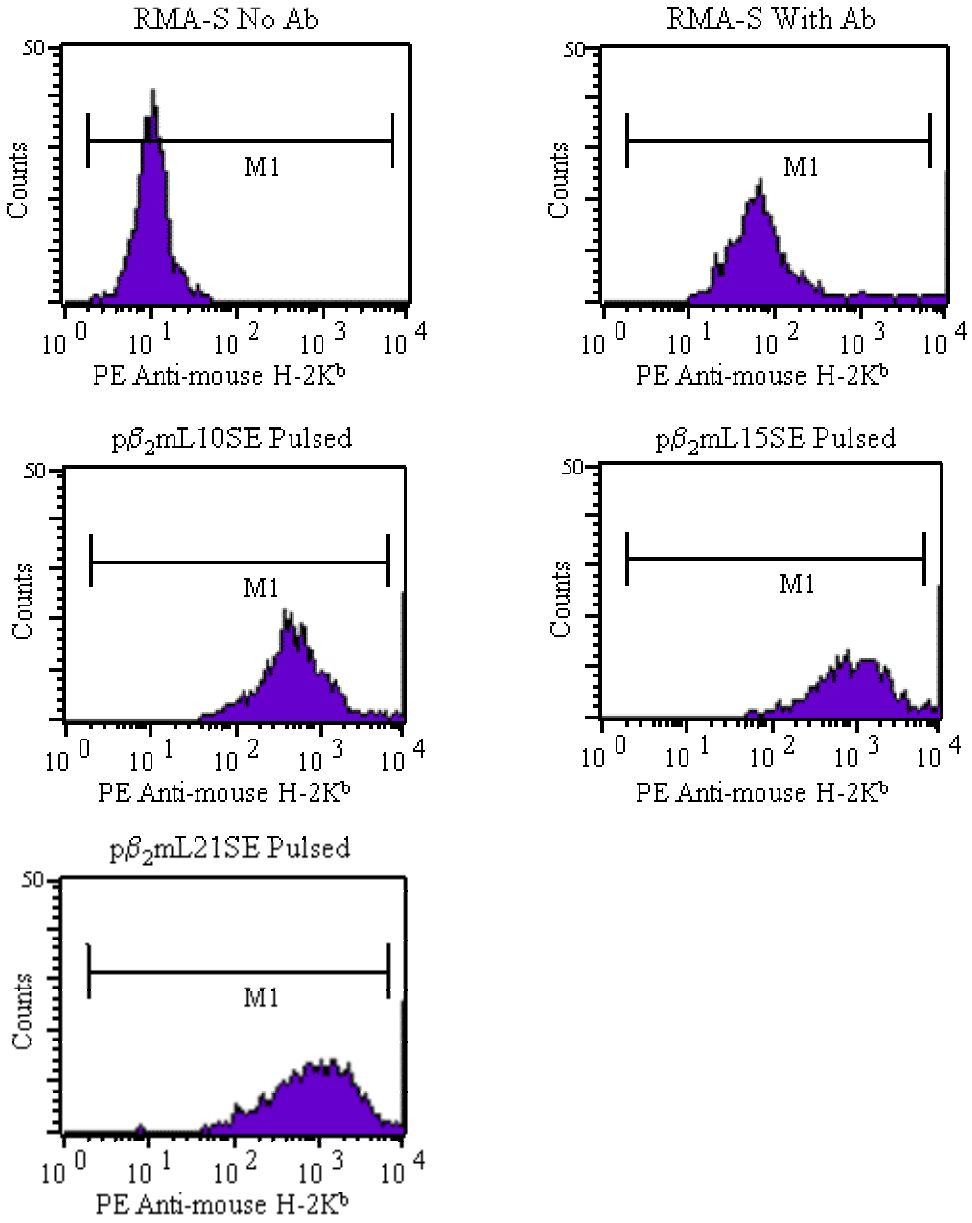
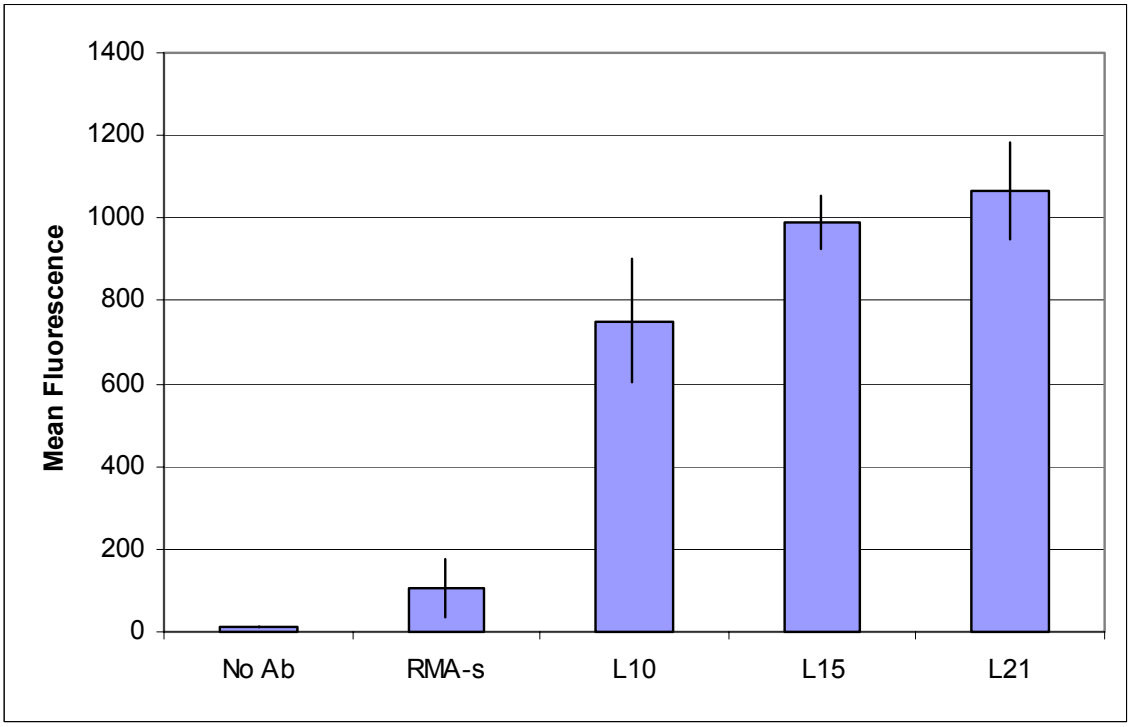


Figure 6.9 Representative anti-mouse H-2K^b stains for MHC-I upregulation

For protocol see Figure 6.8. The M1 gate confines the average mean fluorescence, presented in Figure 6.10, to the peak area.

A



B

	No Ab	RMA-s	L10	L15	L21
No Ab	0.00%	-	-	-	-
RMA-s	98.42%	0.00%	-	-	-
L10	100.00%	99.99%	0.00%	-	-
L15	100.00%	100.00%	93.65%	0.00%	-
L21	100.00%	100.00%	97.47%	64.61%	0.00%

Figure 6.10 MHC-I upregulation experiment summary.

For protocol see Figure 6.8. Each group represents the results of five samples of RMA-S cells pulsed over night with 50 μM of the given β_2m-Sendai epitope fusion protein as designated by the linker size or nothing at all. A) Presents the average mean fluorescence due to PE anti-mouse H-2K^b antibody, with error bars designating the standard deviation. Averages are gated by live cells and by the M1 gate shown in Figure 6.9. B) Gives the percent confidence level that the results are distinct from one another, based on Student t-Test evaluation.

Cells pulsed with the $p\beta_2mL21SE$ showed the greatest mean fluorescence at 1066 ± 116, followed closely by the cells pulsed with $p\beta_2mL15SE$ at 990 ± 62. The cells pulsed with $p\beta_2mL10SE$ had a mean fluorescence of only 753 ± 149. But with 99.99% confidence one can say that the fusion protein causes an upregulation of the MHC-I complex on the surface of the cell as compared to non-pulsed cells, 106 ± 70. The average mean fluorescence of fusion protein pulsed cells was between 7-10 times more than unpulsed cells. Furthermore with close to 95% confidence one can say that the linker sizes of 15 AA or 21 AA performed better than the linker size of 10 AA. At only 65% confidence, with the 21 AA linker being preferred, can one distinguish between the effectiveness of the 15 AA and 21 AA linkers.

MHC EXPRESSION UPREGULATION / STABILIZATION WITH 7AAD

Because of the concern associated with the lack of live cells in the peptide pulsed group from the above MHC-I upregulation experiment, another MHC-I upregulation experiment was conducted. In this experiment 7-Aminoactinomycin D (7AAD) was added. 7AAD is a fluorescent dye that stains dying cells, fluoresces at 646 nM and can be read in channel FL3 of the flow cytometer. Also available at the time of this experiment was the purified $p\beta_2mL15DM$ fusion protein with the dummy epitope. Therefore, an upregulation experiment similar to that described above was performed using 50 μM of purified $p\beta_2mL15DM$ or 50 μM of Sendai epitope. To help link the two experiments together, a group of cells was pulsed with 50 μM of $p\beta_2mL21SE$. The negative controls included RMA-S cells pulsed with nothing that were stained with the MHC-I antibody and 7AAD, with only the MHC-I antibody or with only the 7AAD dye, or nothing at all.

From the lower 7AAD staining seen in the population of cells with the high forward scatter and lower side scatter (gate 1) it is clear that these cells are alive and that the gate used in the previous experiment was correctly around the live cells. Notice increased 7AAD in the cell population with the lower forward scatter and higher side scatter (gate 2) (Figure 6.11). In this experiment there were ample cells in the live cell gate of the peptide pulsed cells, (data not shown). Representative stains demonstrating the shift in fluorescence indicative of the stabilization of the MHC-I molecule are offered in Figure 6.12. The average mean fluorescence for each sample group is presented along with error bars showing the standard deviation (Figure 6.13). The number of samples analyzed for the RMA-S No Ab, RMA-S w/Ab, Dummy, L21, and Peptide were 3,4,3,7, and 8, respectively.

The cells pulsed with the pβ_2mL21SE again showed the highest average mean fluorescence at 201 ± 28, which was approximately 10 times greater than the mean fluorescence of the RMA-S cells stained with antibody, 22 ± 2. The actual mean fluorescence was lower than the previous experiment, but this was simply due to differences in the setting of the voltage or gain used when collecting the data. Both the peptide and the pβ_2mL15DM pulsed cells, at 49 ± 14 and 53 ± 3, respectively, showed a distinct upregulation of the MHC-I molecule when compared to the RMA-S cells. Though the values were less than the pβ_2mL21SE pulsed cells, the mean fluorescence was still twice that of unpulsed cells.

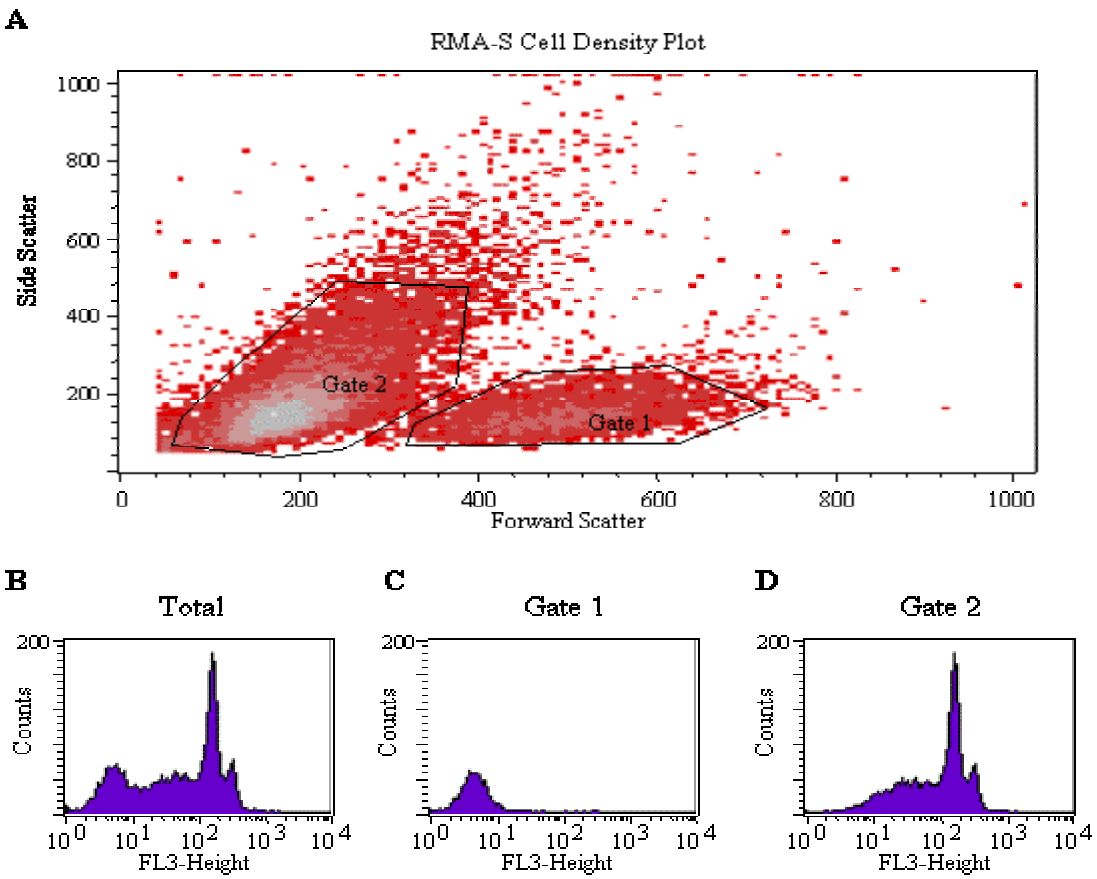


Figure 6.11 RMA-S cell populations stained with 7-Aminoactinomycin D (7AAD).

RMA-S cells ($3.5x10^5$) were grown at 30°C and 5%CO_2 for 1hr. The plate was sealed and the cells were then grown for 18-20hrs at 26°C. The cells were pelleted and washed with wash buffer (PBS with 0.1% sodium azide). Cells were incubated with R-PE Anti-mouse H-2Kb (BD Pharmigen) at 2.0 µg/ml in wash buffer for 30 min on ice. Cells were washed twice and resuspended in 200 µl of was buffer then stored on ice. 4 µl of 7AAD was 10 min before analyzing the fluorescence via FACS. A) Density plot based on forward and side scatter results from un pulsed RMA-S stained with 7AAD. Two cell population have been gated; one cell population, gate 1, has high forward scatter and low side scatter; the other population, gate 2, has a low forward scatter and higher side scatter B) Histogram for total 7AAD fluorescence C) Histogram for 7AAD fluorescence in gate 1 D) Histogram for 7AAD fluorescence in gate 2.

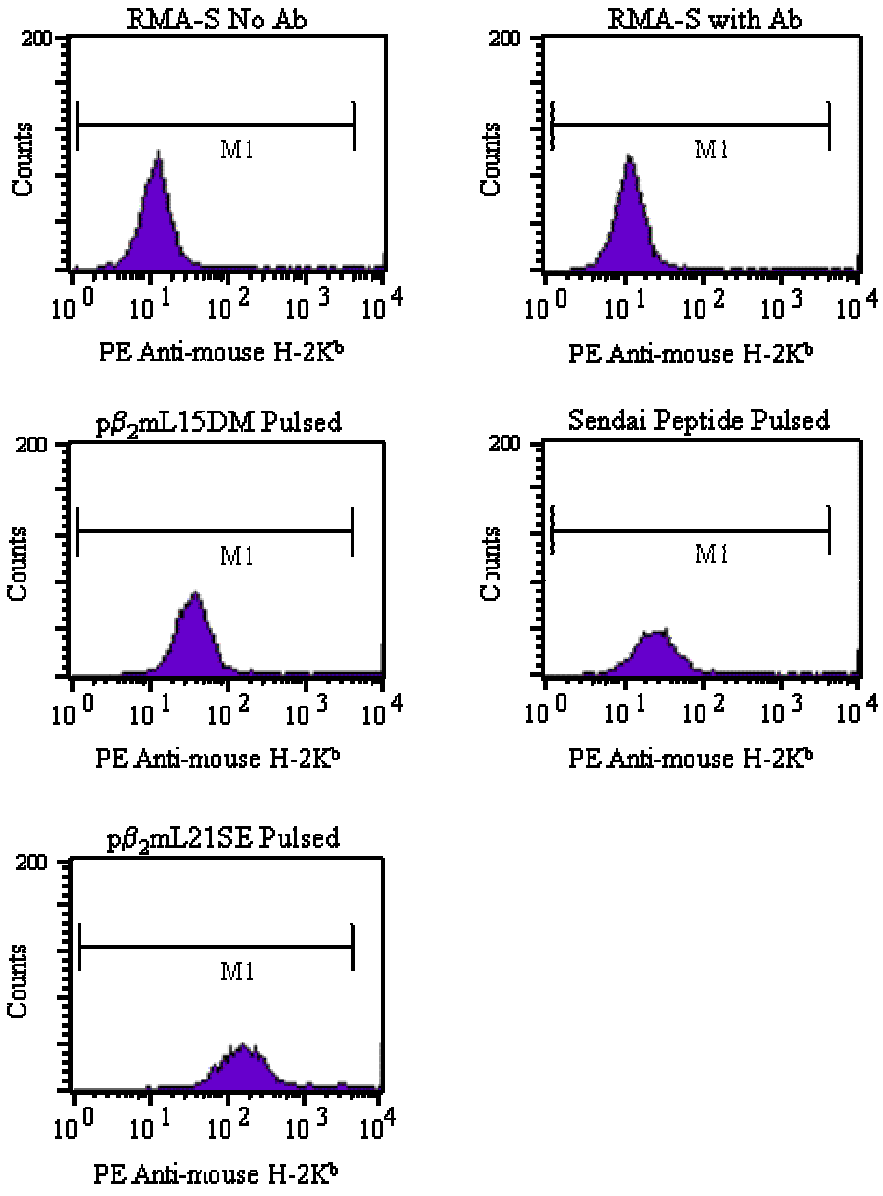
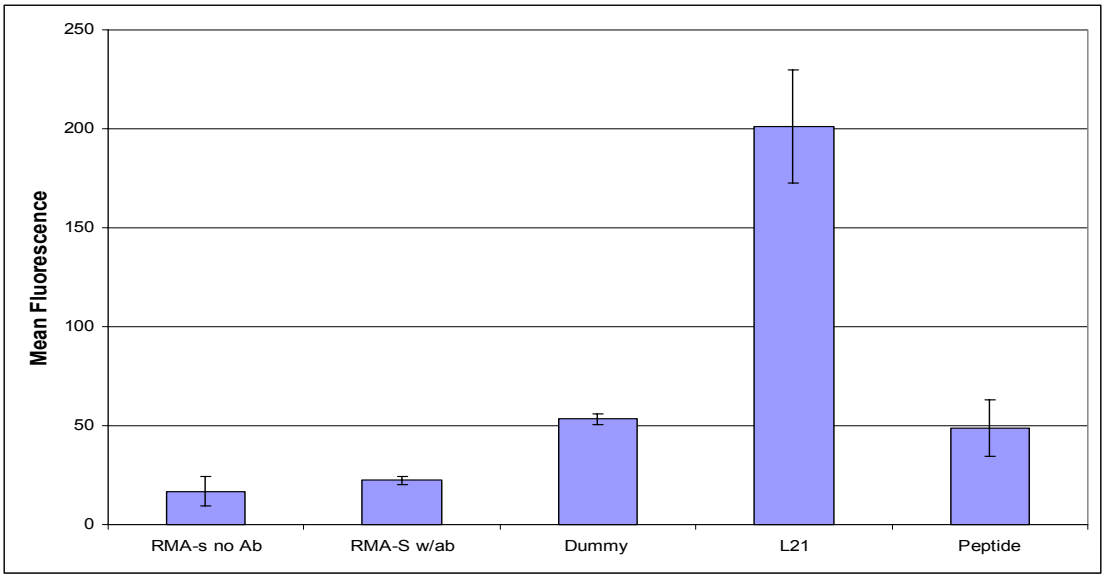


Figure 6.12 Representative anti-mouse H-2K^b stains for MHC-I upregulation, with 7-Aminoactinomycin D (7AAD).

RMA-S after 7-Aminoactinomycin D staining for dead cells. Cells were pulsed with 50 μM pβ_2mL15DM, Sendai peptide, pβ_2mL21SE, or nothing at all. The M1 gate confines the average mean fluorescence, presented in Figure 6.13, to the peak area. For protocol see Figure 6.11

A



B

	RMA-S no Antibody	RMA-S with Antibody	Dummy	L21	Peptide
RMA-S no Antibody	0.00%	-	-	-	-
RMA-S with Antibody	79.90%	0.00%	-	-	-
Dummy	99.87%	100.00%	0.00%	-	-
L21	100.00%	100.00%	100.00%	0.00%	-
Peptide	99.47%	99.54%	38.52%	100.00%	0.00%

Figure 6.13 MHC-I upregulation experiment summary, with 7-Aminoactinomycin D (7AAD).

For protocol see Figure 6.11 A) Presents the average mean fluorescence due to PE anti-mouse H-2K^b antibody, with error bars designating the standard deviation. Averages have been gated by live cells and by the M1 gate shown in Figure 6.12. B) Gives the percent confidence level that the results are distinct from one another, based on Student t-Test evaluation.

T-CELL HYBRIDOMA STIMULATION ASSAY

To determine which linker size would be the best to use as a vaccine, a T-cell hybridoma assay was performed. Sendai specific T-cell hybridomas were obtained from Dr. Woodland's lab at the Trudeau Institute (Cole, Hogg et al. 1995). The ability of the vaccine to trigger a viral-specific immune response *in vitro* was determined by the release of IL-2. Individual T-cell hybridoma clones (M2.5D10, M1.1B3, M2.2E9, or M3.4D3) or a combination of clones (M1.1B3, M2.2E9, and M3.4D3) were cultured with RMA-S cells. The RMA-S cells had been pulsed with the peptide vaccines $p\beta_2$mL10SE, $p\beta_2$mL15SE, or $p\beta_2$mL21SE, or the controls $p\beta_2$mL15DM or Sendai NP peptide. These cells served as the target cells in this assay. There were also two other controls, T-cell hybridomas without RMA-S cells and RMA-S cells without T-cell hybridomas.

The supernatant from these reactions were collected after 24 hours and then transferred to ELISA plates coated with anti-mouse IL-2 antibody (BD Pharmingen). After 2 hours the plates were washed and the secondary biotinylated anti-mouse IL-2 antibody (BD Pharmingen) was added. After 1 hour the plates were again washed and an avidin-horseradish peroxidase conjugate (BD Pharmingen) was added. The reaction was allowed to take place for 30-60 min before being stopped. The absorbance was read at 450 nm and compared against a recombinant mouse IL-2 standard curve.

The results of three different experiments are shown below. Figure 6.14 represents the average of 4 samples of RMA-S cells incubated with M2.5D10, assayed in duplicate. Figure 6.15 depicts the average of 4 samples of RMA-S cells incubated with a combination of clones M1.1B3, M2.2E9 and M3.4D3, assayed in duplicate. Finally, Figure 6.16 depicts the average of 4 samples of RMA-S cells incubated with M1.1B3, M2.2E9 and M3.4D3 individually or as a combination of all three, assayed in duplicate.

In the third experiment the M2.2E9 cells appeared unhealthy prior to the incubation with RMA-S cell and afterwards they looked dead. Also the cells incubated with $p\beta_2$mL15SE and $p\beta_2$mL15DM were found dead. In the third experiment $p\beta_2$mL15SE and $p\beta_2$mL15DM were not concentrated enough so the cells were unable to survive in the diluted media.

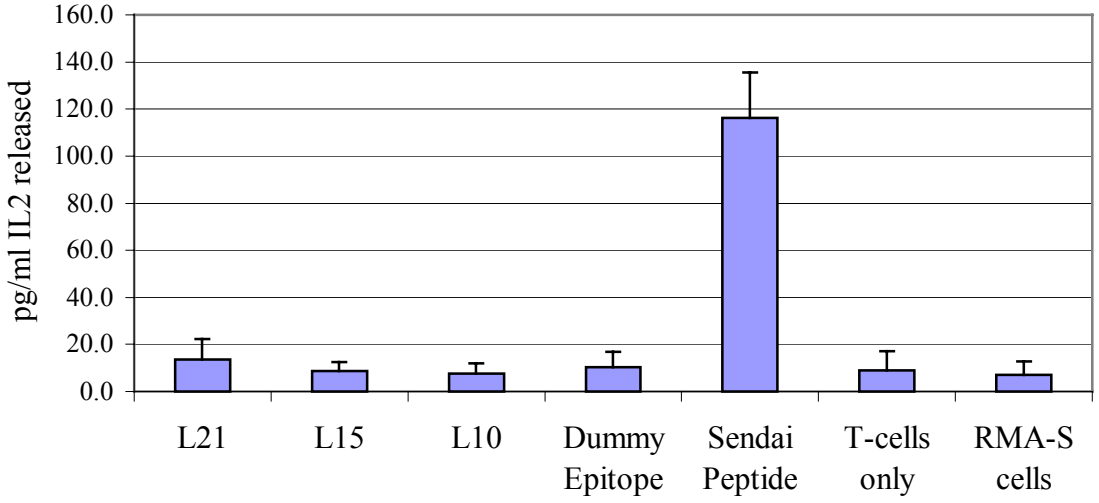


Figure 6.14 T-cell hybridoma stimulation with M2.5D10 clone.

RMA-S cells ($3x10^5$ cells/well) were incubated with 50μM of the vaccine or control at 30°C and 5%CO_2 for 1hr. The plate was sealed and the cells were then grown for 18-20hrs at 26°C. Cells were pelleted and 4.5 $x10^5$ cells of M2.5D10 were added to each well. The RMA-S cells and hybridomas were allowed to react for 18-24 hours at 37°C and 5%CO_2. The supernatant was then assayed for IL-2 using the protocol outlined in the OptEIA Mouse IL-2 Set (BD Pharmingen). Given is the average of 4 samples of RMA-S cells assayed in duplicate. L21, L15 and L10 represent the peptide vaccines with the corresponding linker size. T-cell only and RMA-S cells are the results from unstimulated M2.5D10 alone and RMA-S cells alone, respectively.

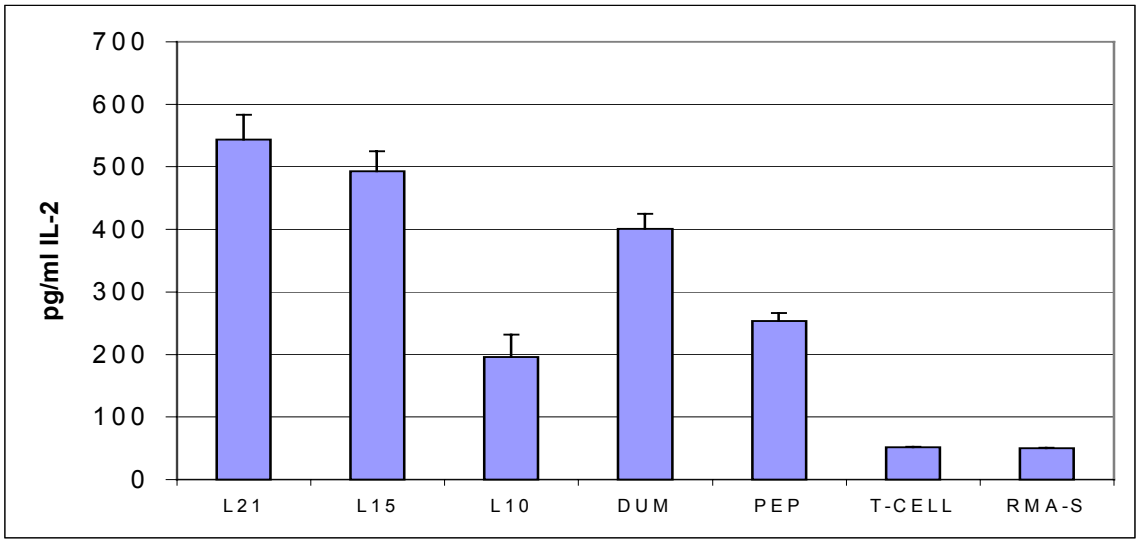


Figure 6.15 T-cell hybridoma stimulation with a mixture of hybridoma clones.

RMA-S cells ($3x10^5$ cells/well) were incubated with 50μM of the vaccine or control at 30°C and 5%CO_2 for 1hr. The plate was sealed and the cells were then grown for 18-20hrs at 26°C. Cells were pelleted and 4.5 $x10^5$ cells consisting of a mixture of M1.1B3, M2.2E9 and M3.4D3 was added to each well. The RMA-S cells and hybridomas were allowed to react for 18-24 hours at 37°C and 5%CO_2. The supernatant was then assayed for IL-2 using the protocol outlined in the OptEIA Mouse IL-2 Set (BD Pharmingen). Given is the average of 4 samples of RMA-S cells assayed in duplicate. L21, L15 and L10 represent the peptide vaccines with the corresponding linker size. DUM signifies the dummy epitope control vaccine and PEP is the results from the Sendai epitope. T-cell and RMA-S are the results from unstimulated T-cell hybridomas and RMA-S cells, respectively.

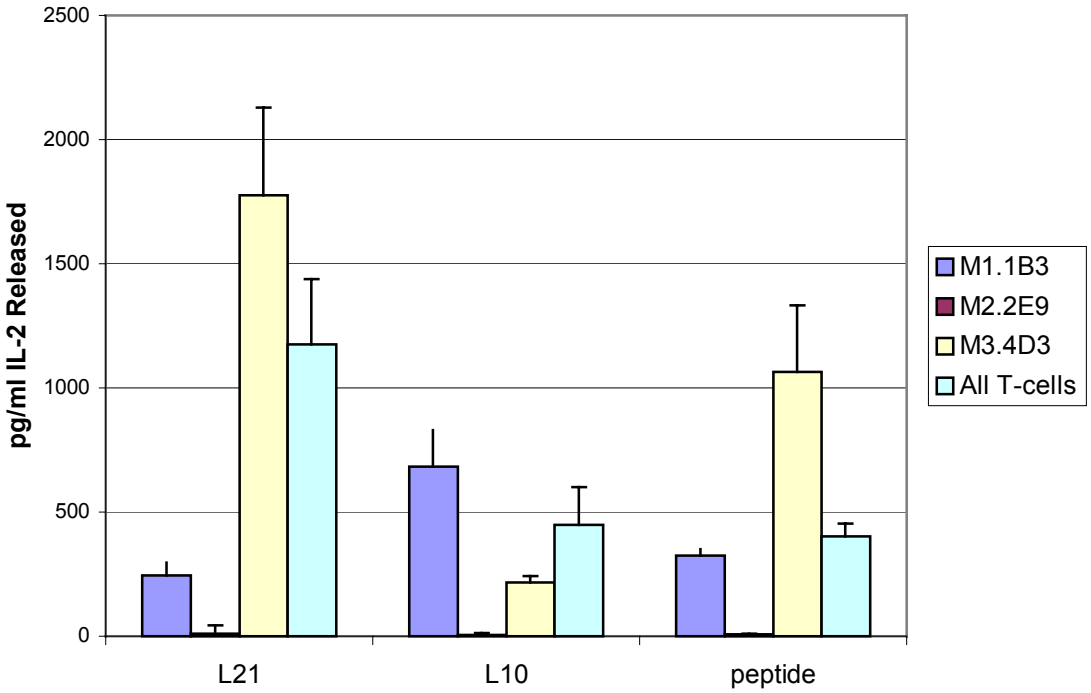


Figure 6.16 T-cell hybridoma stimulation with individual hybridoma clones.

RMA-S cells ($3x10^5$ cells/well) were incubated with 50μM of the vaccine or control at 30°C and 5%CO_2 for 1hr. The plate was sealed and the cells were then grown for 18-20hrs at 26°C. Cells were pelleted and 4.5 $x10^5$ cells of M1.1B3, M2.2E9 and M3.4D3 individually or as a combination of all three, were added. The RMA-S cells and hybridomas were allowed to react for 18-24 hours at 37°C and 5%CO_2. The supernatant was then assayed for IL-2 using the protocol outlined in the OptEIA Mouse IL-2 Set (BD Pharmingen). In this experiment the M2.2E9 cells appeared unhealthy prior to the incubation with RMA-S cell and afterwards they looked dead. The cells incubated with pβ_2mL15SE and pβ_2mL15DM were also found dead. In this experiment pβ_2mL15SE and pβ_2mL15DM were not concentrated enough so the cells were unable to survive in the diluted media. The values represent for samples assayed in duplicate.

The positive result obtained from these experiments was that the peptide vaccines could stimulate viral specific T-cell hybridomas, *in vitro*. The obvious negative conclusion was that the T-cell hybridoma stimulation assay could not be used to determine which linker size to use. The data taken as a whole shows that recognition of peptide vaccines depends on the T-cell hybridoma involved. The M2.5D10 clone, Figure 6.14, was unable to recognize any of the vaccines. The M1.1B3 clone preferentially recognized the pβ_2mL10SE and released 680 pg/ml IL-2 (Figure 6.16). The M3.4D3 clone preferred the pβ_2mL21SE vaccine and secreted 1770 pg/ml IL-2, Figure 6.16. The difference in each clone's ability to recognize the vaccines can be explained by the tolerance they exhibit towards AA substitution in the Sendai epitope sequence. Table 6.1 was complied from data published by Cole, 1995 (Cole, Hogg et al. 1995). The data clearly shows that T-cell hybridoma clones have different tolerance for variations in the wild type Sendai epitope sequence. Notice that the M2.5D10 clone, which was unable to recognize the vaccines, was stringent in its requirement for an alanine residue at position 8. This position is near the C-terminal of the epitope, where the linker attaches in our vaccine candidates.

IN VIVO STIMULATION OF SENDAI SPECIFIC IFN-γ CELLS

Because of the mixed results in distinguishing which linker size was optimal in the T-cell hybridoma stimulation experiment it was determined that the best solution would be to test all the fusion proteins *in vivo*. Therefore, two *in vivo* trials were conducted. The first was a traditional experiment and the second involved a matrix-designed experiment.

Sendai Epitope = F A P G N Y P A L
1 2 3 4 5 6 7 8 9

Hybridoma Clone	F1	G4	N5	A8
M2.5D10	A,D,G,H,I,K,N, L,P,Q,R,S,V,Y	Must be G4	G	E,S,Q
M1.1B3	A,D,Q	H,Y	Must be N5	D,F,H,I,K,N,L, P,Q,R,T,V,Y
M2.2E9	No Data	No Data	No Data	No Data
M3.4D3	No Data	No Data	No Data	No Data

Table 6.1: T-cell hybridoma clone tolerance for variations in the wild type Sendai epitope.

The table was complied from data published by Cole, 1995 (Cole, Hogg et al. 1995). The wild type Sendai epitope AA sequence is given at the top of the table with its corresponding position. Expectable substitution for residues F1, G4, N5 and A8 are given. Unfortunately no data was available for hybridoma clones M2.2E9 and M3.4D3.

Traditional method with peptide vaccines

Five 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were injected intramuscularly (IM) on day 0, 7, and 14 with a solution of TiterMax Gold (CytRx) and either 150 μg of the peptide vaccines, control vaccine, the Sendai epitope or PBS. TiterMax Gold is an adjuvant that the company reports will help facilitate a T_H1 immune response. (From conversations with CytRx technical support) All solutions injected on day 0 and 7, except for the PBS, included 10 μg of the plasmid pUMVC3-mIL12, expressing both the p35 and p40 components of IL-12 (Aldevron). The IL-12 expressing plasmid was not added to any solution on day 14. Spleens and Peyer's patches were isolated 7 days after the last boost and allowed to culture for two days. Cells were counted and assayed for the number of INF-γ producing cells using an ELISPOT assay (BD Pharmingen) (Appendix 1). The number of INF-γ producing cells should be equivalent to the number of cells that recognize the Sendai epitope. Since the epitope is class I MHC restricted the INF-γ producing cells should all be CTLs. The splenocyte response is indicative of the systemic immune response, while the Peyer's patch response represents the mucosal response.

The results show that the peptide vaccines were unable to generate a viral specific CTL response *in vivo* when injected IM along with TiterMax Gold, Table 6.2. A small response was seen in the spleen of mice injected with the Sendai epitope, 11 ± 1.4 IFN-γ producing cells per $2x10^5$ splenocytes. Although, by weight the amount of each injection was similar, by moles the Sendai epitope was approximately 15 times more concentrated. This might suggest that in order to achieve an immune response with the vaccines, mice would have to be injected with over 2 mg per dose. Purifying this amount of vaccine would be difficult. A possible explanation of the poor result could be the use of

TiterMax Gold. According to Weeratna, et. al. 2000 (Weeratna, McCluskie et al. 2000), TiterMax Gold as an adjuvant may have severely shifted the immune system towards a T_H2 response instead of a T_H1. This is contrary to the information provided by the CytRx technical support. An additional problem may have been the route of administration. Since these vaccines require the exchange of the vaccine with native β_2m and peptide on the surface of the cell, perhaps the IM injection site was inadequate to stimulate a response.

Sample	Number of INF-γ producing in 2 x10^5 splenocytes	Number of INF-γ producing in 2 x10^5 Peyer's Patch cells
pβ_2mL21SE (150 μg)	0.8 ± 1.3	0 ± 0
pβ_2mL15SE (150 μg)	2.6 ± 2.7	0 ± 0
pβ_2mL10SE (150 μg)	4.3 ± 4.0	0 ± 0
pβ_2mL15DM (150 μg)	0 ± 0	0 ± 0
Sendai Epitope (150 μg)	11 ± 1.4	0 ± 0
PBS	0 ± 0	0 ± 0

Table 6.2 Average number of INF-γ producing cells ± σ in 2x10^5 splenocytes or Peyer's patches

There were five mice in each group, assayed in duplicate. Mice were vaccinated on day 0, 7, and 14 with 150 μg of vaccine or control in a 1:1 solution with TiterMax Gold. All solutions injected on day 0 and 7, except for the PBS, included 10 μg of the plasmid pUMVC3-mIL12. Spleens and Peyer's patches were isolated and assayed for IFN-γ producing cells using the procedure outlined in the Mouse IFN-γ ELISPOT Set (BD Pharmingen).

'Designed' experiment method with peptide vaccines

The concerns mentioned above were addressed in this experiment. All vaccines were given as a molar amount equivalent to 150 μg of pβ$_2$mL21SE. The TiterMax Gold was removed and injections were performed subcutaneously (SQ) under the arm. The injection site was chosen with hopes that the vaccine would enter the lymph ducts and be taken to either the auxiliary lymph node or brachial lymph node.

The use of designed experiments allows an investigator to determine the effect of multiple variables on the response in the fewest number of experiments. In this experiment the peptide vaccines pβ$_2$mL10SE, pβ$_2$mL15SE, and pβ$_2$mL21SE, and the controls pβ$_2$mL15DM and the Sendai NP peptide, served as the variables. The response measured was the number of INF-γ producing cells determined by an ELISPOT assay. The orthogonal matrix of variables and the average response seen is given in Table 6.3. 7-10 week old C57BL/6 (H-2K^b) (Charles River Laboratories) mice were injected twice-on day 0 and day 14. All injections included 25 μg of the plasmid pUMVC3-mIL12. Spleens and Peyer's patches were isolated 5 days after the boost. Cells were counted and immediately assayed for the number of INF-γ producing cells using an ELISPOT assay (BD Pharmingen). The doses Low (-), Medium (0), and High (+) corresponds to 0 nmoles, 5.4 nmoles, and 10.8 nmoles, respectively. The response represents the average response ± standard deviation (σ) found in two mice assayed twice for the number INF-γ producing cells in 2 x10^5 splenocytes or 2 x10^5 Peyer's patch cells.

	Set-up	$p\beta_2m$ L21SE	$p\beta_2m$ L15SE	$p\beta_2m$ L10SE	$p\beta_2m$ L15DM	Sendai Peptide	Splenocyte Response	Peyer's Response
1	----+	Low	Low	Low	Low	High	0 ± 0.6	0 ± 1.2
2	--++-	Low	Low	High	High	Low	0 ± 1.3	0 ± 1.3
3	-+-+-	Low	High	Low	High	Low	0.25 ± 1.2	0 ± 1.3
4	-++-+	Low	High	High	Low	High	0.75 ± 1.0	0 ± 1.3
5	00000	Medium	Medium	Medium	Medium	Medium	1.0 ± 2.6	0 ± 0.8
6	+--++	High	Low	Low	High	High	0 ± 0.6	0 ± 1.0
7	+-+--	High	Low	High	Low	Low	0 ± 1.2	0 ± 1.7
8	++---	High	High	Low	Low	Low	0 ± 0.8	0 ± 1.0
9	+++++	High	High	High	High	High	4.0 ± 3.0	0 ± 1.0

Table 6.3 Peptide vaccine 'designed' experiment matrix

The vaccines and controls served as the variables and the response was the number of IFN-γ producing cells/ 2 $x10^5$ cells assayed via a standard ELISPOT. Samples were prepared based on a low dose, medium dose, and high dose corresponding to 0 nmoles, 5.4 nmoles, and 10.8 nmoles, respectively. All sample volumes were equalized with PBS. Samples were injected SQ along with 25 μg of pUMVC3-mIL12 on day 0 and 14. Spleens and Peyer's patches were isolated on day 19 and assayed for IFN-γ producing cells using the procedure outlined in the Mouse IFN-γ ELISPOT Set (BD Pharmingen).

The highest number of IFN-γ producing cells, 4.0 ± 3.0 was seen in the 9^{th} set of mice which were injected with a high dose of all the vaccines and controls. This number was very close to the detection limit for this assay, which is advertised at 1/300,000 (Helms, Boehm et al. 2000). This experiment clearly showed that the peptide vaccines, when administered in a matrix format S.C. on day 0 and boosted on day 14, were unable to generate an immune response *in vivo*. The failure to generate IFN-γ producing cells using this mode of administration triggered the termination of the peptide version as a primary vaccine. The use of the $p\beta_2$mL21SE version as a boost after DNA vaccination was explored below.

β_2m-Sendai Epitope Vaccine as a Mammalian Expression Vector

DNA VACCINE CONSTRUCT DESIGN AND CLONING

The design of the β_2-microglobulin-Sendai epitope DNA vaccine involved fusion of β_2m to the same Sendai epitope ($NP_{324-332}$) and then to an endoplasmic reticulum signal peptide. In order for the Sendai epitope to load correctly into the MHC heavy chain a spacer linking the N-terminal of β_2-microglobulin to the C-terminal of the Sendai epitope was required. The optimal length of the linker was unknown so three linker sizes were chosen: 10, 15 and 21 amino acids. As before the linker was composed of glycines and serines, which should be non-immunogenic and provide steric flexibility. The signal peptide sequence (MARSVTLVFLVLVSLTGLYA, Swiss-protein P01887) was the normal signal sequence for mouse β_2m and should guide the translating protein to the endoplasmic reticulum. DNA constructs with 10 and 15 AA linkers were constructed with one method while the cloning of the DNA construct with the 21 AA linker proceeded in a slightly different fashion. Negative control vaccines with a dummy epitope or no epitope were engineered in the same method as the 21 AA linker DNA vaccine.

Construct design and cloning of β_2-microglobulin-sendai epitope- signal peptide DNA vaccine (β_2mSESP) with a 10 or 15 amino acid linker

The same pBluescript-SK vector coding for the murine β_2-microglobulin, mentioned above, acted as the initial template in a three-step extension PCR. To clone the β_2mSESP DNA vaccine, three upstream primers (Sigma Genosys) were used in

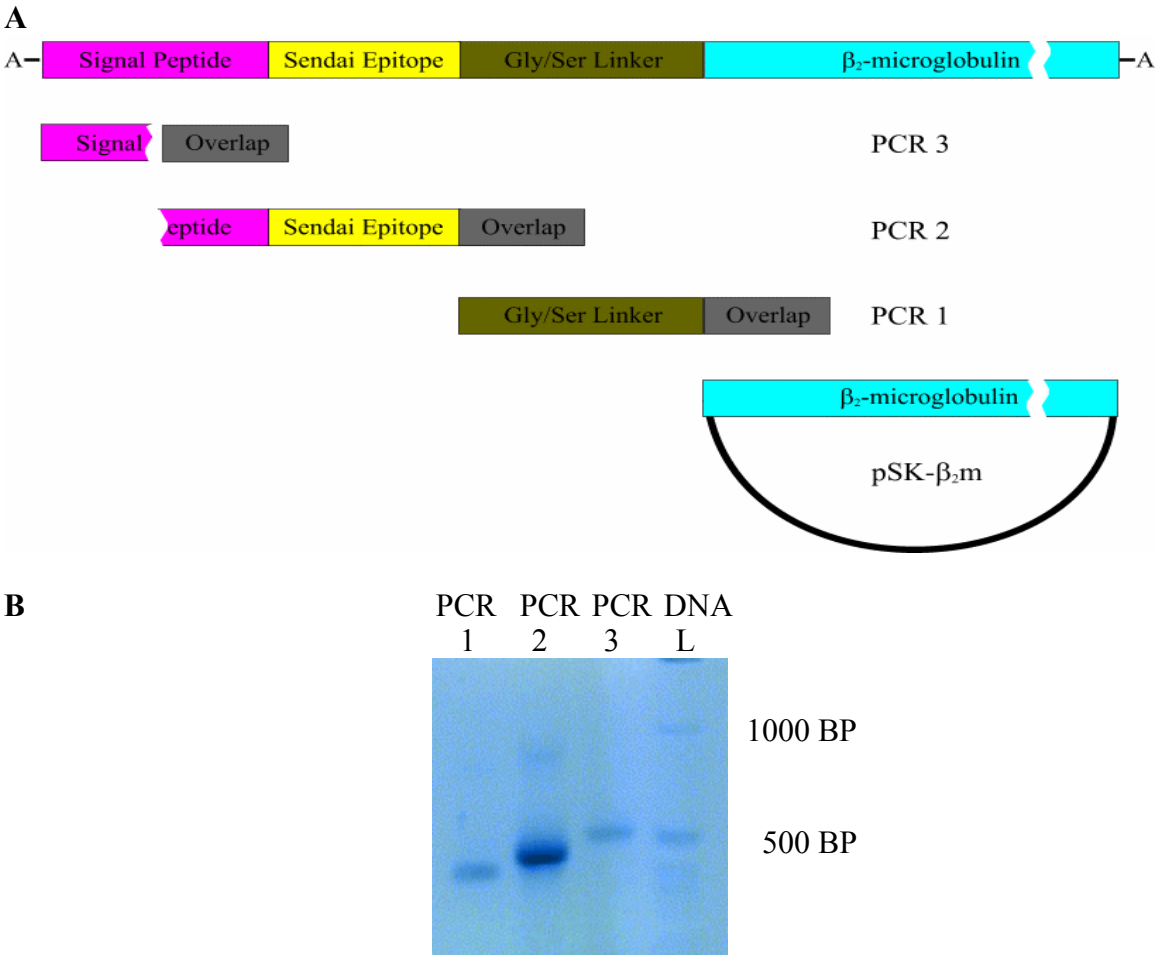


Figure 6.17 DNA vaccine construct.

A) Schematic depiction of the extension PCR used to constructed the β_2mL10SEP and β_2mL15SESP DNA vaccines. The first extension PCR adds the linker to the N-terminal of β_2m; the second PCR adds the Sendai epitope and part of the wild type mouse signal peptide for β_2m; the final PCR adds the remainder of the signal peptide. B) A color-inverted digital image of a 1% agarose gel showing the increase in the β_2mL10SESP construct size from PCR1 to PCR2 then again in PCR3. The downstream primer and upstream primers were at a final concentration of 0.3μM. The PCR reaction was set at 94.0°C for 5 min, then cycled for 30 times from 94.0°C for 45s -> 55.0°c for 45s -> 72.0°C for 60s. The template for the first reaction was the pSK-β_2m plasmid, while the templates for the second and third PCR reactions were the gel-purified product from the previous PCRs.

three sequential PCR reactions to add the necessary sequences to the β_2m gene. For each PCR reaction, the T7 terminator primer was used as the downstream primer. The first extension PCR primer added either the 10 AA or 15 AA linker to the β_2m gene. The second primer added the Sendai epitope and a portion of the signal peptide. The final PCR primer, added the remainder of the signal peptide and a Kozak initialization signal (Figure 6.17).

Endonuclease restriction sites were also added at both ends of the PCR product during the extension PCR reactions: Nhe I and AFL II were added just upstream of the signal peptide and sites for Xho I, Apa I, and Kpn I were already downstream of the transcription stop site. The flexibility to add new pathogenic epitopes to the vaccine was integrated into the construct by surrounding the region coding for the Sendai epitope with Age I and BamH I restriction sites.

The final PCR products and the plasmid pVAX1 (Novagen) were cut with the restriction enzymes Nhe I and Xho I. The digested pVAX1 vector was treated with 1 unit of shrimp alkaline phosphatase to remove terminal phosphates. The PCR product was then ligated into the pVAX1 plasmid and the resulting ligation was transformed into TOP10F′ cells. The cells were plated on LB/Agar plates contain kanamycin. Colonies were chosen and then screened for the insert using PCR (Figure 6.18). The plasmid was isolated from the appropriate colonies and the sequence verified at the University of Texas Sequence Center. Figures 6.19 and 6.20 give the construct DNA sequence and protein sequence for β_2ML10SESP and β_2ML15SESP, respectively. Highlighted are the various segments of the construct, such as the signal peptide, the Sendai epitope, the linker and the murine β_2m. Restriction digestion sites are underlined.

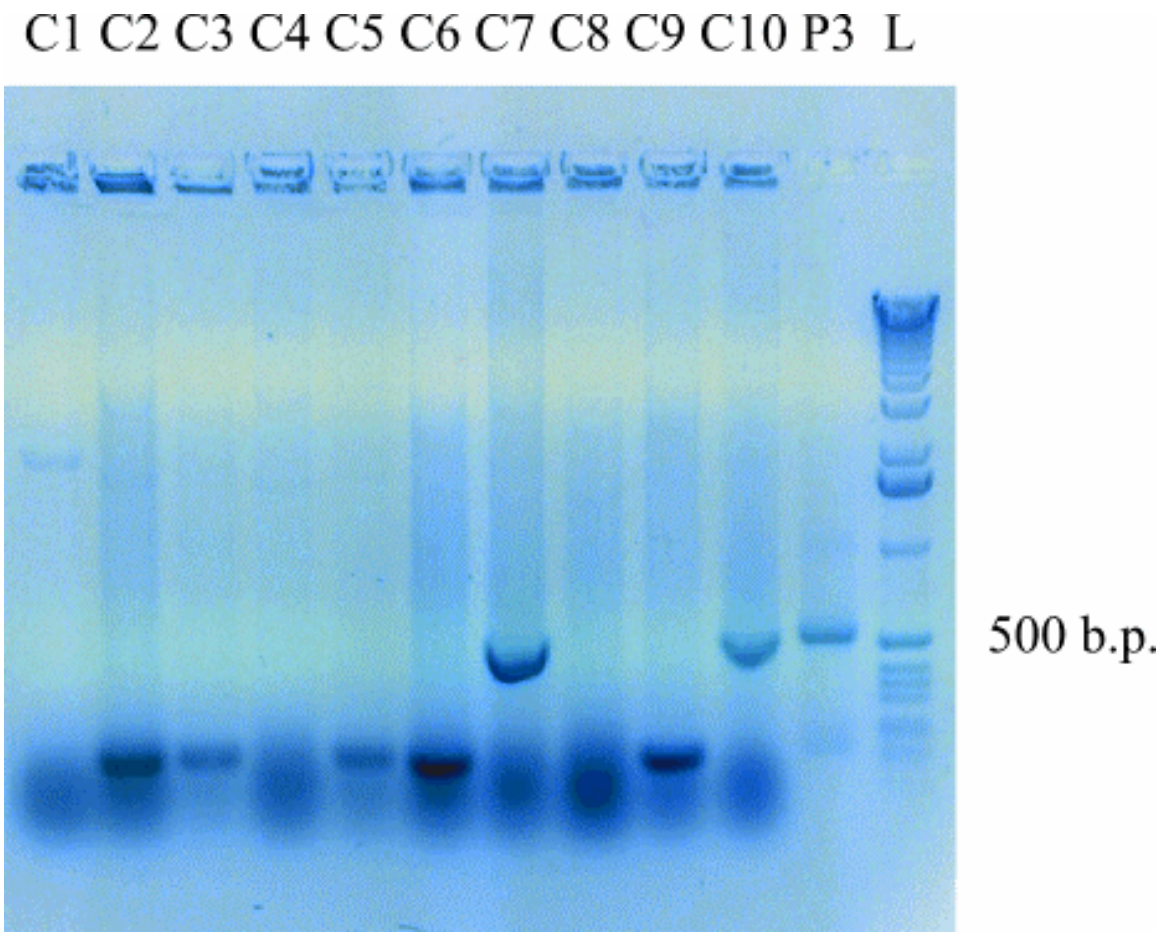


Figure 6.18 Colony PCR screen for β_2ML10SESP.

A color-inverted digital image of a 1% agarose gel with the products of a colony PCR screen for β_2ML10SESP. β_2ML10SESP was transformed into TOP10F' cells by heat shock at 42°C for 45 seconds. Cells were allowed to recover in SOC for 1 hour and then plated on LB plates containing 50 μg/ml kanamycin. With a toothpick individual colonies were chosen and then screen by PCR for the insert. C1-C10 correspond to the 10 colonies selected. P3 is the purified product from the third extension PCR for β_2ML10SESP. L is a DNA ladder.

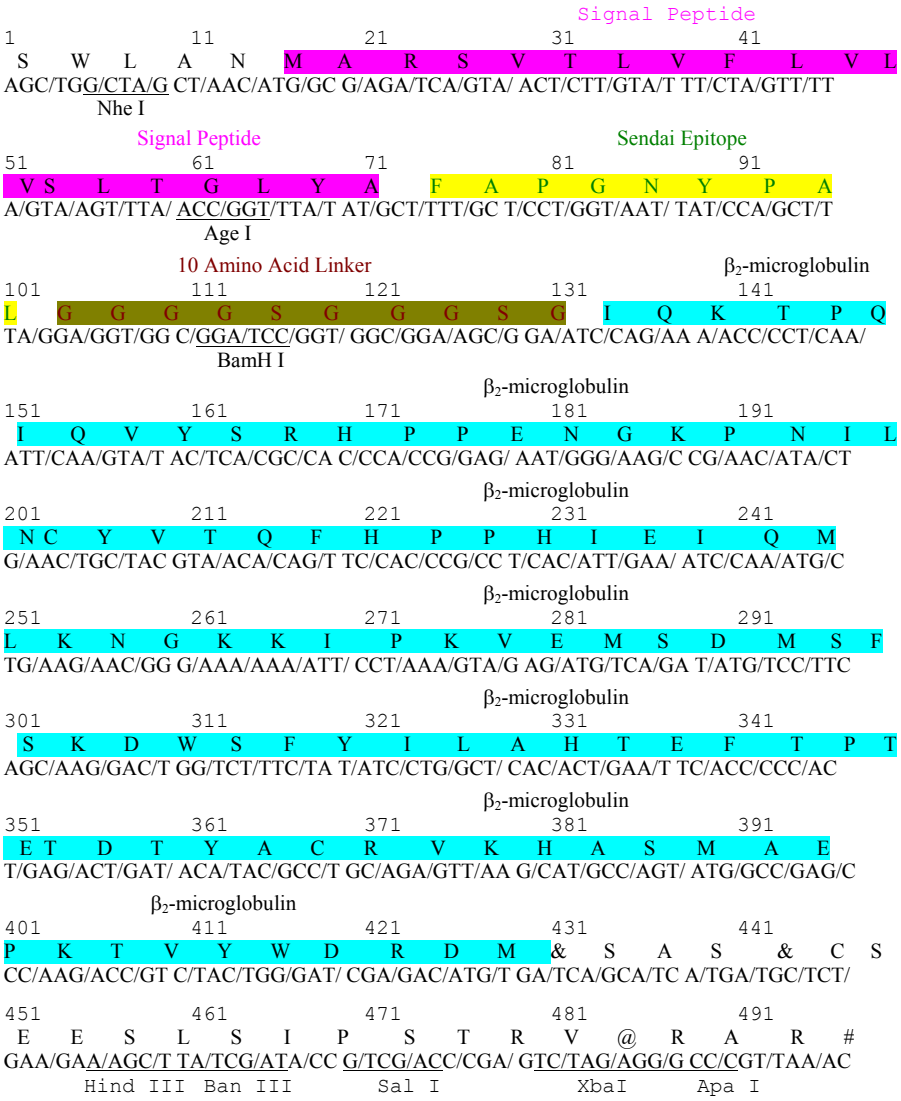


Figure 6.19 The sequence for β_2mL10SESP.

TOP10F' cells were transformed by heat shock with pVAX1 β_2mL10SESP and grown overnight in 5mls of LB with 50 μg/ml kanamycin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the reverse primer. The sequence is reported in the 5'-3'direction.

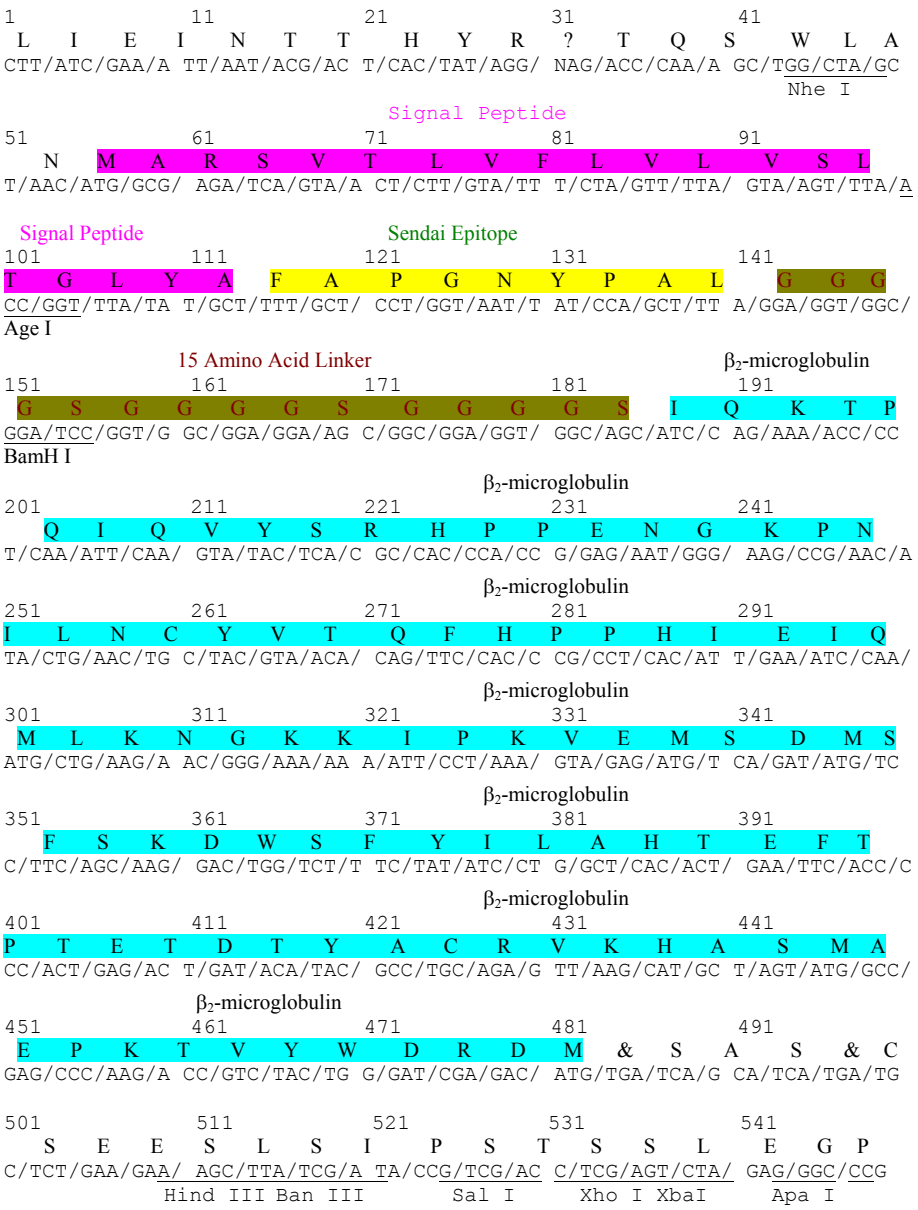


Figure 6.20 The sequence for β_2mL15SESP.

TOP10F' cells were transformed by heat shock with pVAX1 β_2mL15SESP and grown overnight in 5mls of LB with 50 μg/ml kanamycin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the reverse primer. The sequence is reported in the 5'-3'direction.

Construct design and cloning of β_2-microglobulin-sendai epitope- signal peptide DNA vaccine with a 21 amino acid linker (β_2mL21SESP) and the control vaccines with a dummy epitope (β_2mL21DMSP) or no epitope (β_2mL21NOSP).

The β_2mL21SESP vaccine and the control vaccines with a dummy epitope (β_2mL21DMSP) or no epitope (β_2mL21NOSP) were created in the same manner. The process began by digesting pVAX1 β_2mL15SESP first with the endonucleases Age I and then with BamH I. This digestion cut out a small portion of the signal peptide, the Sendai epitope, and a small portion of the linker.

Next, complementary oligonucleotides (Sigma Genosys) that encoded for the lost portion of the signal peptide, the Sendai epitope, and the lost portion of the linker plus 6 additional amino acids were annealed together. When annealed the oligonucleotides formed overhangs corresponding to the overhangs of Age I and BamH I. The annealed oligonucleotides were then ligated into the cut pVAX1 β_2mL15SESP. The ligation product was transformed into TOP10F′ cells and then plated on LB/Agar plates containing kanamycin. Isolated colonies were picked and then screened for the insert using PCR. (Data not shown) The plasmid was isolated from the appropriate colonies and the sequence verified at the University of Texas Sequence Center. Figure 6.21 gives the construct DNA and protein sequences for β_2ML21SESP.

The only difference between the creation of β_2mL21SESP and β_2mL21DMSP or β_2mL21NOSP was the sequence of the complementary oligonucleotides used. The oligonucleotides for β_2mL21DMSP encoded for the dummy epitope (FASIRALAL) instead of the Sendai epitope (Figure 6.22). The oligonucleotides for β_2mL21NOSP completely left the Sendai epitope out (Figure 6.23). The creation of these vaccines demonstrated the functionality incorporated in the design of the DNA vaccine to switch epitopes.

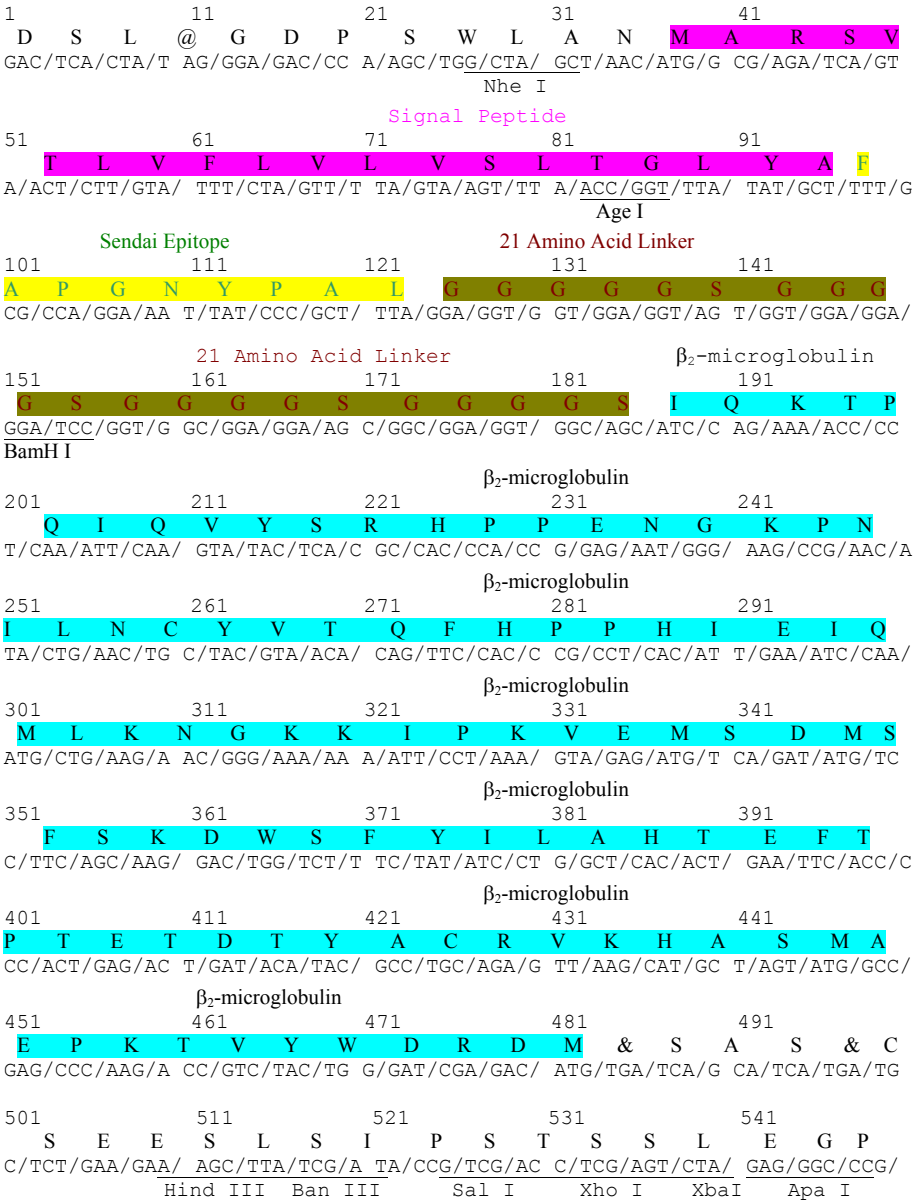


Figure 6.21 The sequence for β_2mL21SESP.

TOP10F' cells were transformed by heat shock with pVAX1 β_2mL21SESP and grown overnight in 5mls of LB with 50 μg/ml kanamycin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the reverse primer. The sequence is reported in the 5'-3'direction.

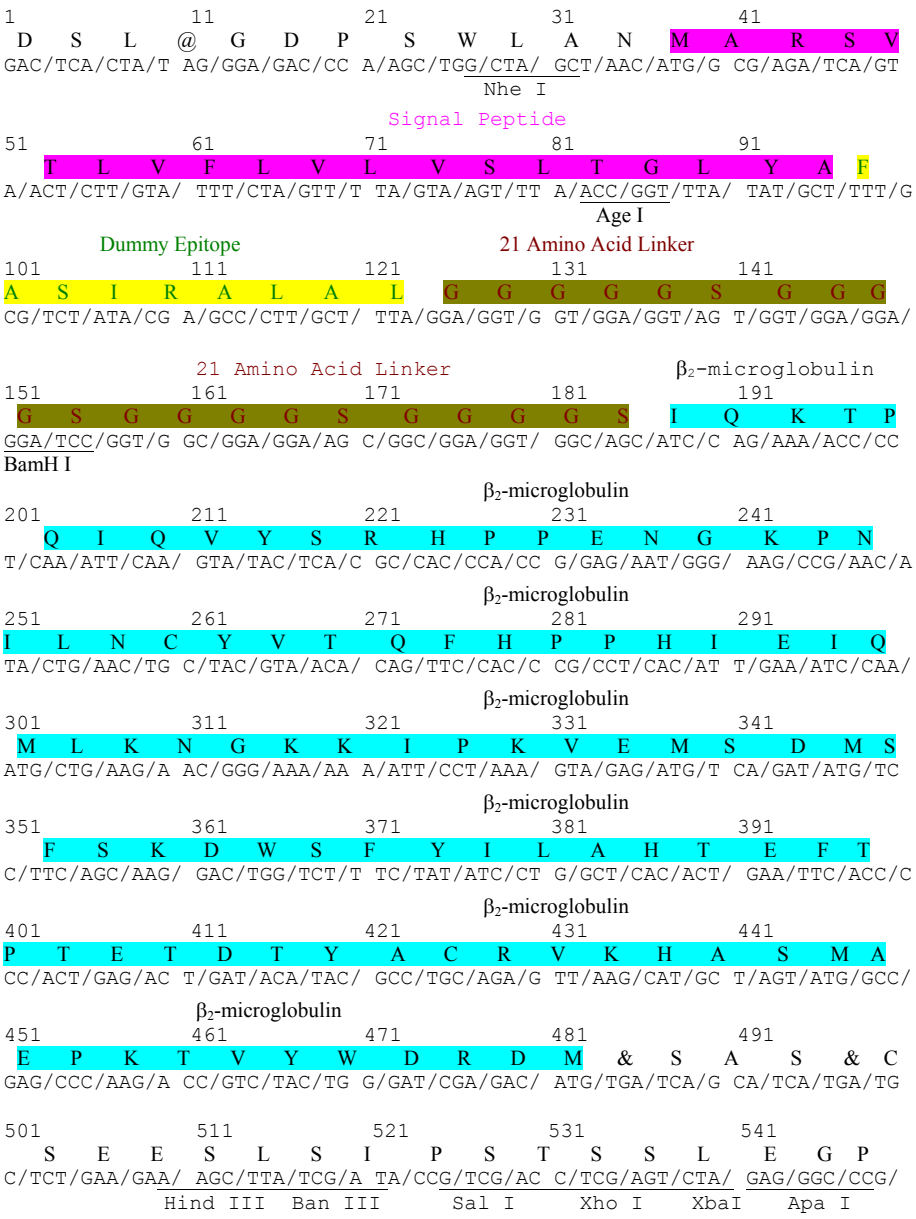


Figure 6.22 The sequence for β_2mL21DMSP.

TOP10F' cells were transformed by heat shock with pVAX1 β_2mL21DMSP and grown overnight in 5mls of LB with 50 μg/ml kanamycin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the reverse primer. The sequence is reported in the 5'-3'direction.

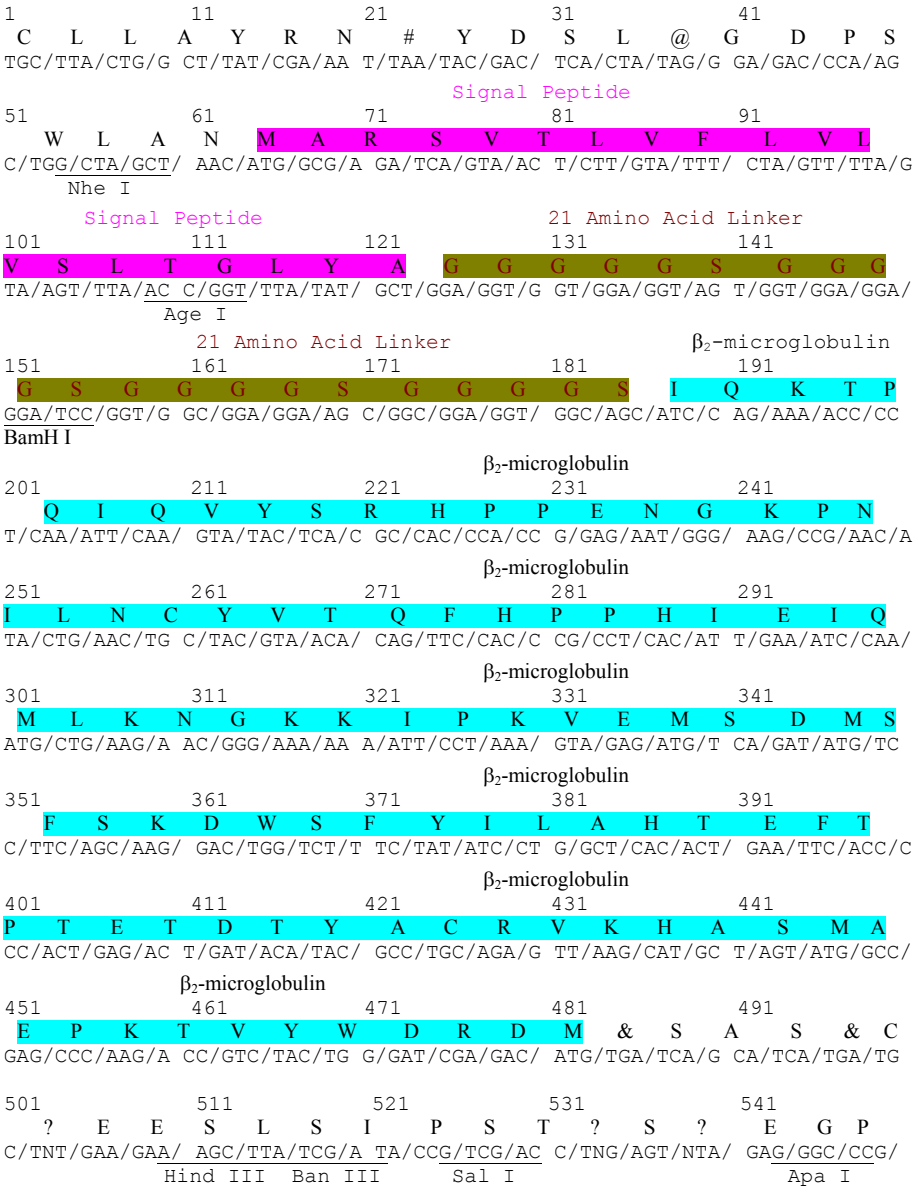


Figure 6.23 The sequence for β_2mL21NOSP.

TOP10F' cells were transformed by heat shock with pVAX1 β_2mL21NOSP and grown overnight in 5mls of LB with 50 μg/ml kanamycin. The plasmid was isolated using the Mini Prep kit from Qiagen. The plasmid was then sent to the University of Texas Sequence Center and the sequence was verified using the reverse primer. The sequence is reported in the 5'-3'direction.

T-CELL HYBRIDOMA STIMULATION ASSAY

The T-cell hybridoma stimulation assay was first attempted with RMA-S cells acting as the target. Attempts to transect this cell line with a green fluorescence protein expressing plasmid, peGFP-N2 (CLONTECH) failed. Thus, the RMA-S cell line was abandoned for the NOR-10 cell line (ATCC, CCL-197). The NOR-10 cell line is derived from the abdominal wall muscle of C57BL/10 mice. Transfection of the NOR-10 cell line proved possible using the SuperFect transfection reagent (Invitrogen). Using a flow cytometer to determine GFP expression, these cells demonstrated transfection efficiencies between 6-9%. (Data not shown)

NOR-10 cells were then transfected using the SuperFect transfection protocol (Appendix 1) with peGFP-N2 and either the DNA vaccines β_2mL10SESP, β_2mL15SESP, or β_2mL21SESP or the controls β_2mL21DMSP, β_2mL21NOSP, or pVAX1. The peGFP-N2 plasmid was used in this experiment to verify transfection and to normalize the results. The transfected NOR-10 cells then served as the target cells in the hybridoma assay.

Individual T-cell hybridomas clones M1.1B3 and M3.4D3 or a combination of the two were cultured with transfected NOR-10 cells. The supernatant from these reactions was collected after 24 hours and then transferred to ELISA plates coated with anti-mouse IL-2 antibody (BD Pharmingen). After 2 hours the plates were washed and the secondary biotinylated anti-mouse IL-2 antibody (BD Pharmingen) was added. After 1 hour the plates were again washed and an avidin-horseradish peroxidase conjugate (BD Pharmingen) was added. The reaction was allowed to take place for 30-60 minutes before being stopped. The absorbance was read at 450 nm and compared against a

recombinant mouse IL-2 standard curve. Figure 6.24 presents the average of four samples assayed in duplicate.

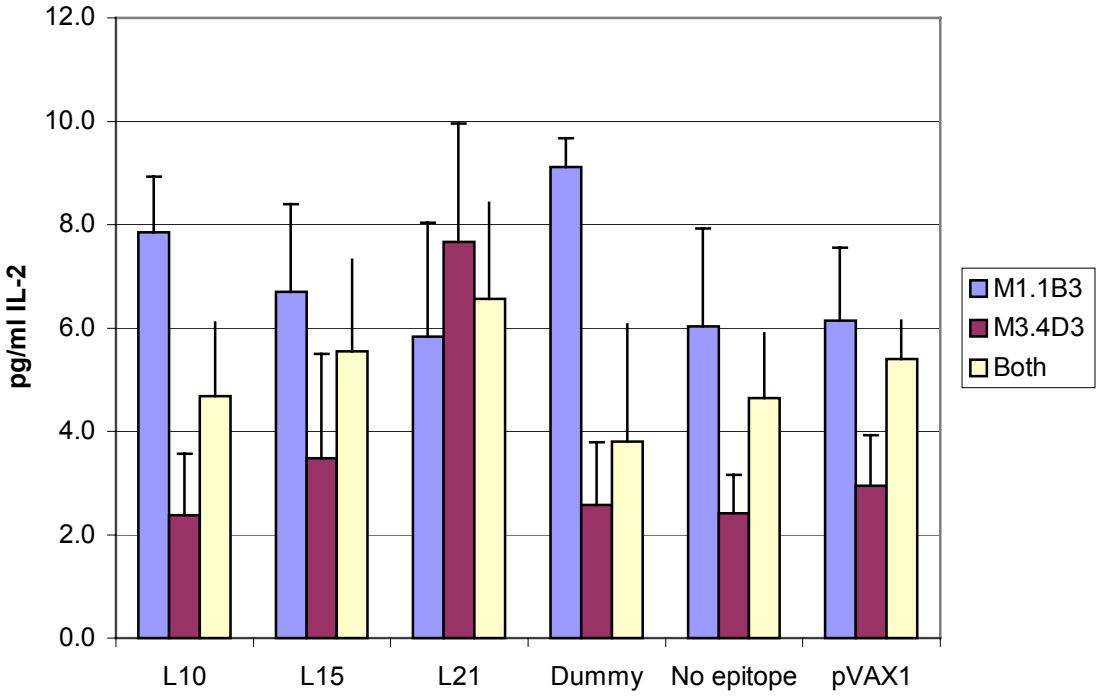


Figure 6.24 T-cell hybridoma stimulation assay results for the DNA vaccines.

L10, L15 and L21 correspond to the linker size of the vaccine used. The controls Dummy, No epitope and pVAX1 correspond to β_2mL21DMSP, β_2mL21NESP and the pVAX1 vector, respectively. NOR-10 cells ($2x10^6$) were plated the night before they were transfected with 9 μg of DNA vaccine and 3 μg of peGFP-N2 using the SuperFect transient transfection protocol (Invitrogen) at a 1 μg of DNA to 5 μl of SuperFect ratio. Cells were grown overnight and then passed into a 12 well plate at $3.5x10^5$ cells per well. These cells were allowed to adhere overnight and then incubated with $1x10^6$ T-cell hybridomas for 18-24 hours. The release of IL-2 was measured by ELISA. These results have not been normalized for transfection efficiency. The error bars represent the standard deviation.

The results presented in Figure 6.24 were similar to results observed on two other attempts to stimulate the hybridomas with transfected NOR-10 cells. The greatest release of IL-2 observed came from M1.1B3 cells incubated with NOR-10 cells that had been transfected with β_2mL21DMSP at 9.1 pg/ml and the next highest response was given by those transfected with β_2mL10SESP at 7.9 pg/ml. For the M3.4D3 hybridoma, the greatest release of IL-2 was seen by cells incubated with NOR-10 cells that had been transfected with β_2mL21SESP at 7.7 pg/ml. Given the generally low values, high σ, and the high value for pVAX1, it is questionable whether any of data were far enough above the signal to noise ratio to be considered real values. These data were disappointing, however an inability to stimulate hybridoma cells with a transfected cell line *in vitro*, should only be taken as that and not viewed as a strong indicator of what would be expected *in vivo*.

IN VIVO DNA STUDIES

'Designed' experiment method with DNA vaccines

The use of designed experiments allows an investigator to determine the effect of multiple variables on the response in the fewest number of experiments. In this experiment the DNA vaccines β_2mL10SESP, β_2mL15SESP, and β_2mL21SESP and the controls β_2mL21DMSP, β_2mL21NOSP and the pVAX vector served as the variables. The response measured was the number of INF-γ producing cells that recognize the Sendai epitope as determined by an ELISPOT assay. The orthogonal matrix of variables and the average response seen is given in Table 6.4. 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were injected twice - on day 0 and day 14. All injections included 25 μg of pUMVC3-mIL12 and were done IM, splitting the sample

equally between both hind legs. The dose Low (-), Medium (0), and High (+) corresponds to 0 pmoles, 26.1 pmoles, and 52.4 pmoles, respectively. Spleens and Peyer's patches were isolated 5 days after the second injection. Cells were counted and immediately assayed for the number of INF-γ producing cells using an ELISPOT assay (BD Pharmingen). The response represents the average number of spots ± σ found in two mice assayed twice for number INF-γ producing cells in $2x10^5$ splenocytes. Similar to the results achieved with the peptide vaccine there was no measurable INF-γ producing cells in the Peyer's patches and therefore it was not presented.

	Set-up	β_2m-L21SESP	β_2m-L15SESP	β_2m-L10SESP	β_2m-L21DMSP	β_2m-L21NOSP	PVAX	Splenocyte Response
1	----++	Low	Low	Low	Low	High	High	0.3 ± 0.5
2	--++--	Low	Low	High	High	Low	Low	1.3 ± 1.5
3	-+-+-+	Low	High	Low	High	Low	High	1.3 ± 1.9
4	-++-+-	Low	High	High	Low	High	Low	17.8 ± 7.5
5	000000	Medium	Medium	Medium	Medium	Medium	Medium	10.5 ± 1.7
6	+--++-	High	Low	Low	High	High	Low	14.5 ± 16.6
7	+-+--+	High	Low	High	Low	Low	High	32.0 ± 2.6
8	++----	High	High	Low	Low	Low	Low	16.0 ± 9.6
9	++++++	High	High	High	High	High	High	46.0 ± 43.0

Table 6.4 DNA vaccine 'designed' experiment matrix

The vaccines and controls served as the variables and the response was the number of IFN-γ producing cells/ $2x10^5$ cells assayed via a standard ELISPOT. Samples were prepared based on a low dose, medium dose, and high dose corresponding to 0 pmoles, 26.1 pmoles, and 52.4 pmoles, respectively. All sample volumes were equalized with PBS. Samples were injected IM along with 25 μg of pUMVC3-mIL12 on day 0 and 14. Spleens and Peyer's patches were isolated on day 19. No response was observed in the Peyer's patches and therefore the data was not presented.

According to the central limit theorem, the averages from sample sets of any population will be distributed approximately normally around the population average. The experiment was designed so that four groups of mice were given each variable and another four groups of mice were not injected with that variable. Thus an easy way to get an idea of the effect of each variable is to subtract the average response of mice without the variable from the average response of mice that received the variable, Table 6.5. Conservatively the two variables with the greatest effect were β_2mL21SESP and β_2mL10SESP.

	β_2mL21SESP	β_2mL15SESP	β_2mL10SESP	β_2mL21DMSP	β_2mL21NOSP	PVAX
Mice with	6,7,8,9	3,4,8,9	2,4,7,9	2,3,6,9	1,4,6,9	1,3,7,9
Mice w/o	1,2,3,4	1,2,6,7	1,3,6,8	1,4,7,8	2,3,7,8	2,4,6,8
positive	27.1	20.3	24.3	15.8	19.6	19.9
negative	5.1	12.0	8.0	16.5	12.6	12.4
Effect (INF-γ producing cells in $2x10^5$ splenocytes)	22.0	8.3	16.3	-0.8	7.0	7.5
INF-γ producing cells in $1x10^6$ splenocytes	110	41.5	81.5	-4.0	35	37.5

Table 6.5 Analysis of the effect each variable contributed in the DNA vaccine 'designed' experiment.

Mice with and mice w/o correspond to the group numbers that contained or did not have the variable, respectively. Positive and negative are the sum of the number of spots with or with out the variable, respectively. The effect is the result of subtracting the negative from the positive.

A more sophisticated method of interpreting the data would be to develop a model. Using the Design Ease 6.0.9 "designed experiment" software package the data was modeled with all the variables to give the following equation:

.

$$\text{IFN-}\gamma \text{ producing cells} = -14.0 + 4.2\times10^{11}*(\beta_2\text{mL21SESP}) + 1.6\times10^{11}*(\beta_2\text{mL15SESP}) + 3.1\times10^{11}*(\beta_2\text{mL10SESP}) - 1.4\times10^{10}*(\beta_2\text{mL21DMSP}) + 1.3\times10^{11}*(\beta_2\text{mL21NOSP}) + 1.4\times10^{11}*(\text{pVAX})$$

The variables are given in moles and the standard error for each coefficient is 1.0×10^{11}. The model had a Model F-value of 5.01, which implies the model is significant and that there is only a 0.13% chance that a "Model F-Value" this large could occur due to noise. The F distribution compares the ratio of the model mean square to the error mean square, thus the higher the F-value the better the model fits the data. The coefficients represent the relative effect of each of the variables. Although the model itself lacks noise, there appears to be great variability between animals as shown by the intercept of –14.0 and the standard error in the coefficient. Even considering these factors, at higher than 68% confidence level the β_2mL21SESP vaccine has a greater effect in the model than the β_2mL15SESP vaccine and the controls β_2mL21DMSP, β_2mL21NOSP and the pVAX1 vector. At the 68% confidence level there is no significant difference between β_2mL21SESP and β_2mL10SES (Figure 6.25)

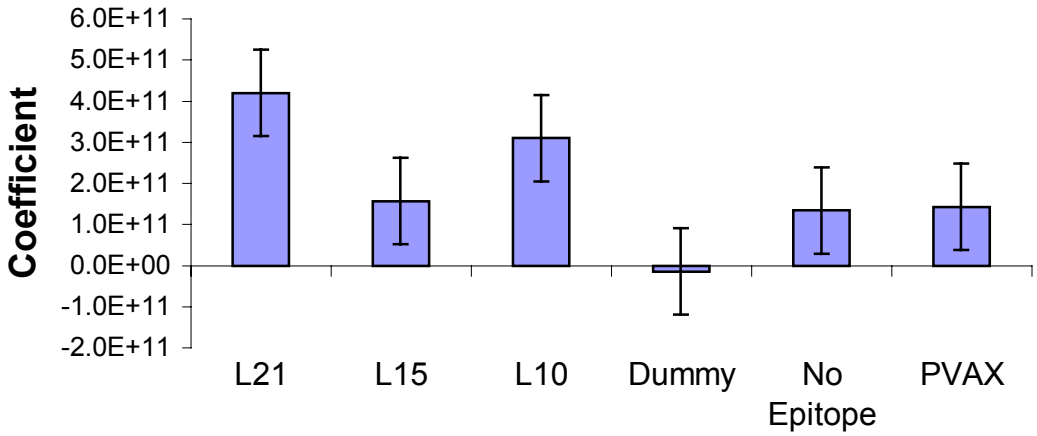


Figure 6.25 The model coefficients for the DNA vaccine designed experiment.

The experimental procedure is described table 6.4. The error bars are set at one standard error, representing the 68% confidence level.

Traditional method with DNA vaccines

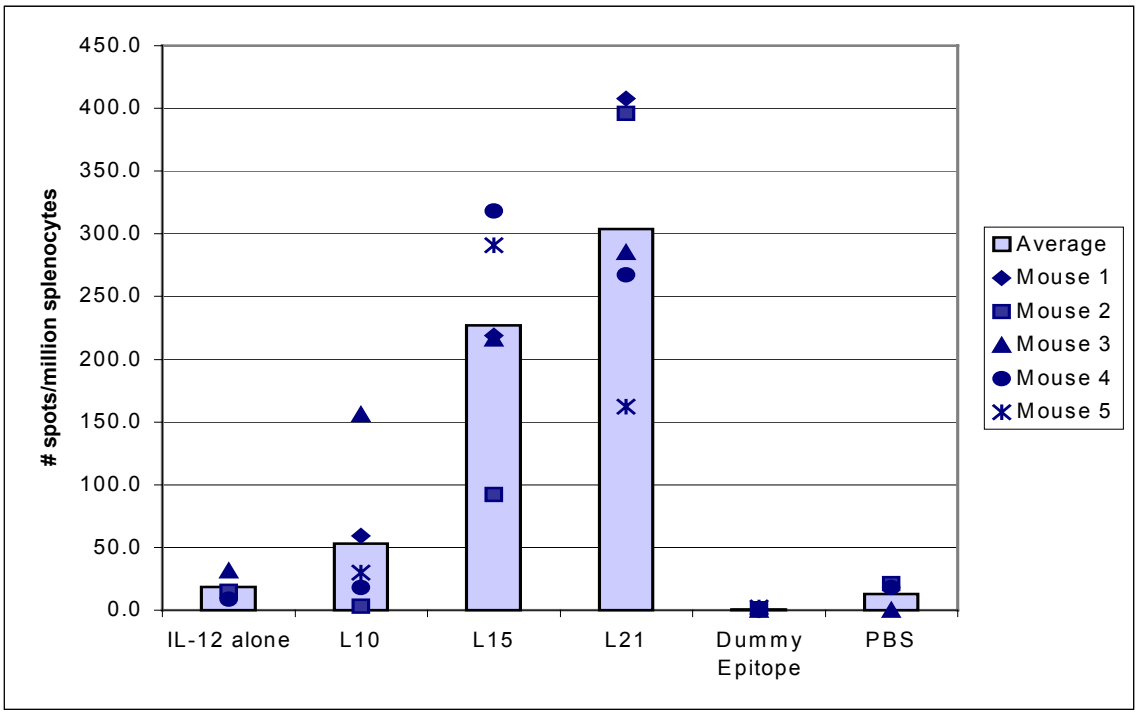
A very positive result from the experiment described above was the observation of INF-γ producing cells, which clearly demonstrated that the vaccines generated a viral specific immune response. Because of the variability shown in the model and the difficulty of publishing data from designed experiments in animals, a more traditional study was also performed. In this experiment, twenty-six 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were divided into 4 groups of 5 and 2 groups of 3. Groups of 5 mice were injected IM, as before, into the hind leg on day 0 and 14 with 25 μg of pUMVC3-mIL12 and 92.1 pmoles (~200 μg) of either β_2mL10SESP, β_2mL15SESP, β_2mL21SESP or the control β_2mL21DMSP. Groups of 3 were injected IM on day 0 and 14 with either 25 μg pUMVC3-mIL12 or PBS. Spleens were isolated 14

days after the last injection. Cells were counted and immediately assayed for the number of INF-γ producing cells using an ELISPOT assay (BD Pharmingen) (Figure 6.26a).

The DNA vaccine with a 21 AA linker gave the highest number of INF-γ producing cells, at 304 ± 102 spots/million splenocytes, followed closely by the 15 AA linker at 227 ± 88 spots/million splenocytes. The 10 AA linker vaccine gave a value of only 53 ± 61 spots/million splenocytes. The dummy epitope and PBS controls gave values of 1 ± 1 spots/million splenocytes and 13 ± 11 spots/million splenocytes, respectively. Clearly from this data the β_2m-linker-Sendai epitope-signal peptide DNA vaccines were able to generate viral specific CTLs.

Student t-Tests were performed to determine if the values obtained were significantly different from one another (Figure 6.26b). The values given in a matrix format correspond to the percent confidence that the value for the vaccine at the top of the column was different than the value for the vaccine at the right of each row. I can say with almost 100% confidence for mice vaccinated with either β_2mL21SESP or β_2mL15SESP and with 82% confidence for mice vaccinated with β_2mL10SESP that these vaccinated mice gave a higher response than mice vaccinated with IL-12 along. In addition, with over 90% confidence I chose the β_2mL21SESP vaccine to further characterize.

A



B

	IL-12 alone	L10	L15	L21	Dummy Epitope	PBS
IL-12 alone	0.00%	-	-	-	-	-
L10	81.64%	0.00%	-	-	-	-
L15	100.00%	99.99%	0.00%	-	-	-
L21	100.00%	100.00%	90.72%	0.00%	-	-
Dummy Epitope	99.91%	98.86%	100.00%	100.00%	0.00%	-
PBS	40.62%	86.67%	100.00%	100.00%	93.21%	0.00%

Figure 6.26 Traditional determination of DNA vaccine linker size.

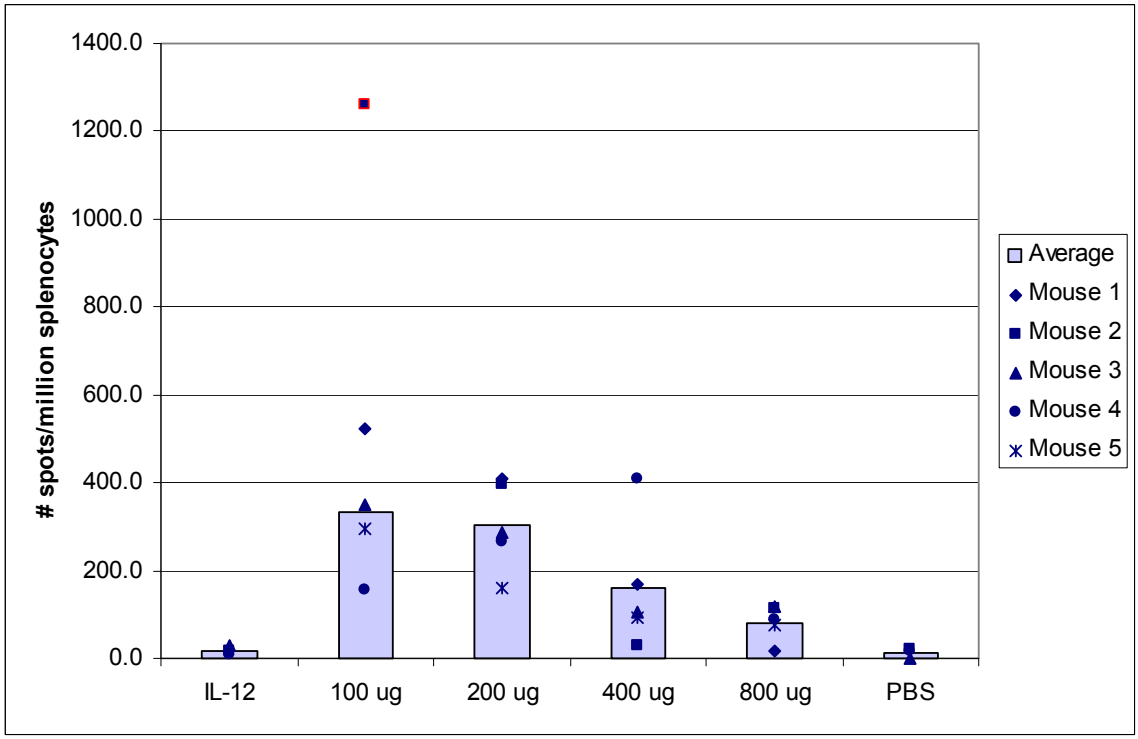
There were five C57BL/6 mice used in groups L10, L15, L21, and Dummy Epitope, and 3 mice in the IL-12 and PBS controls. L10, L15, and L21 correspond to the linker size of the vaccine. A) Depicts graphically the average value for the ELISPOT assay for each group and the average of duplicate ELISPOT assays for each mouse in the group. B) List the confidence levels, based on Student t-Test analysis of each group's ELISPOT data, at which one can say the values were statistically different.

Dose response

In this experiment, twenty-six 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were divided into 4 groups of 5 and 2 groups of 3. The groups of five were injected IM, into each hind leg as before on day 0 and 14 with 25 μg of pUMVC3-mIL12 and either 100 μg, 200 μg, 400μg, or 800 μg of β_2mL21SESP. The groups of three received either 25 μg of pUMVC3-mIL12 or just PBS on days 0 and 14. Spleens were isolated 14 days after the last injection. Cells were counted and immediately assayed for the number of INF-γ producing cells using an ELISPOT assay (BD Pharmingen). Figure 6.27 depicts the results of this experiment. The outlier value, highlighted in red, of 1260 spots for mouse 2 in the 100 μg group, was excluded based on the results of a Q test at 90% confidence (Q = 0.668). This value was not included in the average for the 100 μg group or in the Student t-Test analysis.

Contrary to what was expected, doses over 200 μg, when given on day 0 and again as a boost on day 14, generated fewer viral-specific splenocytes than lower doses. There was little difference between the number of spots seen in mice vaccinated with 100 μg, at 332 ± 151 spots/million splenocytes and mice vaccinated with the 200 μg, at 304 ± 102 spots/million splenocytes. However, when mice were vaccinated with 400 μg of vaccine the number of spots dropped into half to 162 ± 148 spots/million splenocytes. Then as the dose was doubled to 800 μg the number of spots was cut in half again to 82 ± 40 spots/million cells. With over 97% confidence I can say that the values for the 100 μg and 200 μg dose were different that the values for the 400 μg or 800 μg dose. Also with almost 90% confidence the 400 μg dose was statistically different than the 800 μg dose.

A



B

	IL-12	100 μg	200 μg	400 μg	800 μg	PBS
IL-12	0.00%	-	-	-	-	-
100 μg	99.98%	0.00%	-	-	-	-
200 μg	100.00%	35.48%	0.00%	-	-	-
400 μg	96.90%	97.46%	97.86%	0.00%	-	-
800 μg	99.81%	99.99%	100.00%	89.08%	0.00%	-
PBS	40.62%	99.98%	100.00%	97.37%	99.87%	0.00%

Figure 6.27 Dose response for β_2mL21SESP.

There were five C57BL/6 mice used in groups 100 μg, 200 μg, 400 μg, and 800 μg, and 3 mice in the IL-12 and PBS controls. The value, highlighted in red, of 1260 spots for mouse 2 in the 100 μg group, was thrown out based on the results of a Q test at 90% confidence. This value was not included in the average for the 100 μg group for the determination of the confidence level. A) Depicts graphically the average value for the ELISPOT assay for each group and the average of duplicate ELISPOT assays for each mouse in the group. B) Lists the confidence levels, based on Student t-Test analysis of each groups ELISPOT data, at which one can say the values were statistically different.

Duration of immune response

In this experiment, twenty-five 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were divided into 3 test groups of 5 and 2 control groups of 5. The test groups of five were injected IM into each hind leg on days 0 and 14 with 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12. The control groups received either 25 μg of pUMVC3-mIL12 and PBS or just PBS on days 0 and 14. Test mice were sacrificed 7, 14, and 28 days after the boost, corresponding to days 21, 28, and 42, respectively. One mouse from each control group was sacrificed on day 21; three from each group were sacrificed on day 28; and one from each group was sacrificed on day 42. Spleens were isolated; cells were counted and immediately assayed for the number of INF-γ producing cells using an ELISPOT assay. Figure 6.28 shows that the number of IFN-γ producing cells decreased as the time from boost increased. But even at the furthest day from the boost examined, 28 days, the splenocytes from vaccinated mice showed significantly more INF-γ producing cells, 70 ± 36 spots/million splenocytes, than either the IL-12 control or PBS control, which showed 1 and 0 spots/million splenocytes, respectively.

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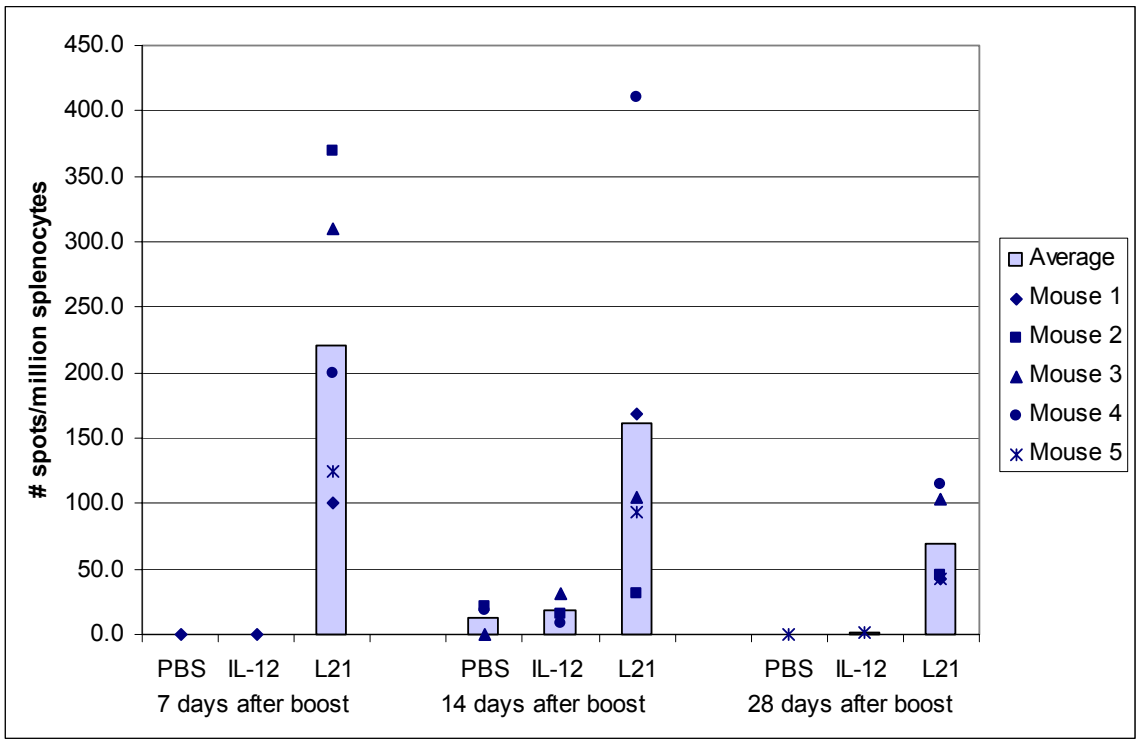

B

	7 Days After Boost	14 Days After Boost	28 Days After Boost
7 Days After Boost	0.00%		
14 Days After Boost	67.62%	0.00%	
28 Days After Boost	99.90%	93.50%	0.00%

Figure 6.28 Duration of the immune response after the boost.

Results from an ELISPOT assay for INF-γ producing splenocytes in C57BL/6 mice vaccinated on day 0 and boosted on day 14. L21 corresponds to five mice dosed with 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12. Mice were sacrificed 7, 14, or 28 days after the boost. IL-12 refers to mice primed and boosted only with 25 μg of pUMVC3-mIL12. PBS refers to mice primed and boosted only with PBS. For the IL-12 and PBS samples there was only 1 mouse on day 7 and 28 and 3 mice on day 14. A) Depicts graphically the average value for the ELISPOT assay for each group and the average of duplicate ELISPOT assays for each mouse in the group. B) Lists the confidence levels, based on Student t-Test analysis of each group's ELISPOT data, at which one can say the values were statistically different.

IL-12 effect on ELISPOT

To determine if the IL-12 adjuvant improved the vaccine's ability to generate viral-specific splenocytes sixteen 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were divided into 2 groups of 5 and 2 groups of 3. The groups of five were injected IM, into each hind leg on days 0 and 14 with 400μg of β_2mL21SESP and either 0 mg or 25 μg of pUMVC3-mIL12. The groups of three received either 25 μg of pUMVC3-mIL12 or just PBS on days 0 and 14. Figure 6.29 gives the results of this experiment. With almost 98% confidence there was a difference between the number of spots produced by mice treated with the IL-12 plasmid in addition to the vaccine and those treated with only the vaccine. Based on the average response the use of IL-12 as an adjuvant increases the number of viral specific spleen CTLs by 4 fold: from 42 ± 40 spots/million splenocytes seen in vaccinations without IL-12 to 162 ± 148 spots/million splenocytes seen when IL-12 was added. With only 85% confidence can it be stated that the mice vaccinated with the DNA vaccine without IL-12 generated more spots than the controls of IL-12 alone at 19 ± 12 spots/million splenocytes or PBS at 13 ± 11 spots/million splenocytes.

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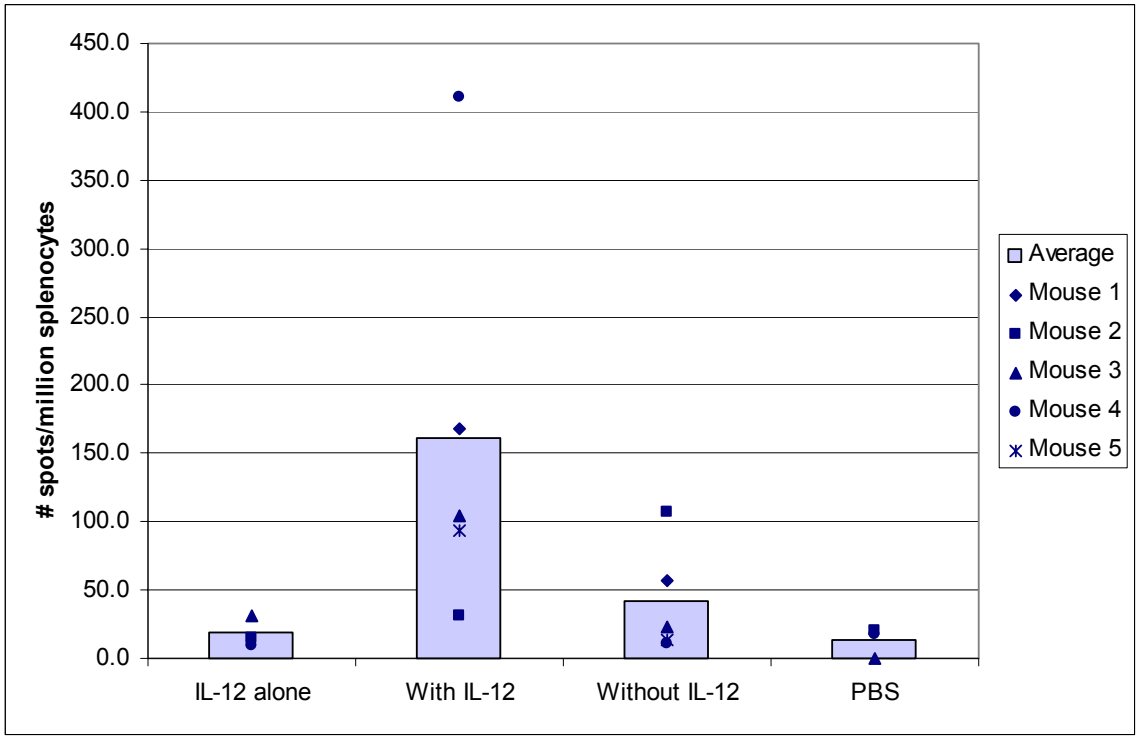

B

	IL-12 alone	With IL-12	Without IL-12	PBS
IL-12 alone	0.00%	-	-	-
With IL-12	96.90%	0.00%	-	-
Without IL-12	82.54%	97.93%	0.00%	-
PBS	40.62%	97.37%	88.84%	0.00%

Figure 6.29 The effect of IL-12 on IFN-γ producing splenocytes.

ELISPOT assay to determine the effect of IL-12 as an adjuvant on the number of IFN-γ producing cells generated in the spleen. 400 μg of β_2mL21SESP was injected IM into 5 mice on days 0 and 14 either with 25 μg of pUMVC3-mIL12 or without pUMVC3-mIL12. There where 3 mice in the control groups IL-12 alone and PBS. A) Depicts graphically the average value for the ELISPOT assay for each group and the average of duplicate ELISPOT assays for each mouse in the group. B) Lists the confidence levels, based on Student t-Test analysis of each group's ELISPOT data, at which one can say the values were statistically different.

Protein vaccine as a boost

Other labs have shown that DNA vaccination followed by a boost with the recombinant protein could increase the specific immune response (Letvin, Montefiori et al. 1997; Habel, Chanel et al. 2000; Hammond, Jansen et al. 2001; Hevey, Negley et al. 2001). To test this idea with our vaccination strategy, sixteen 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were divided into 2 test groups of 5 and 2 control groups of 3. The tests groups were injected IM into each hind leg on day 0 with 400μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12. On day 14 one test group received 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 administered IM and the other receive 400 μg of pβ_2mL21SE and 25 μg of pUMVC3-mIL12 administered SQ. The groups of three received IM either 25 μg of pUMVC3-mIL12 or just PBS on day 0 and 14. Figure 6.30 gives the results of this experiment. At only a 74% confidence level was there a difference between the average number of spots produced by mice treated with the booster of recombinant fusion protein on day 14, 226 ± 92 spots/million splenocytes, compared to a boost with the DNA construct, 162 ± 148 spots/million splenocytes. Both groups were significantly higher than either of the controls.

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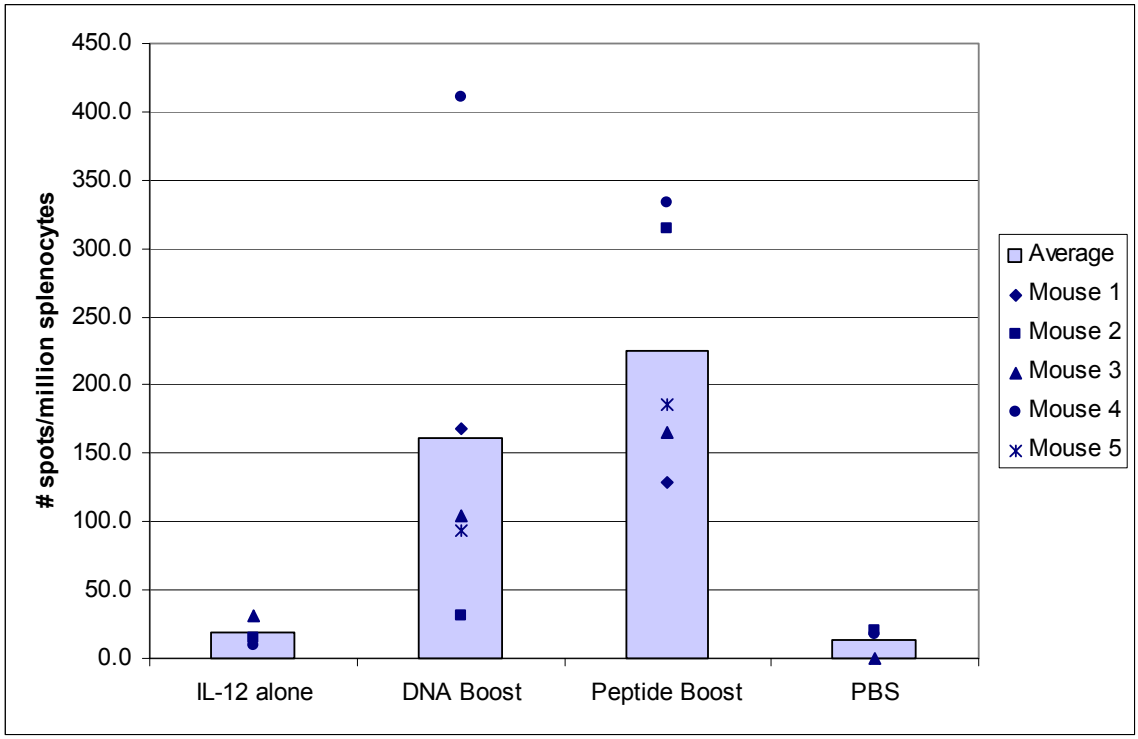

B

	IL-12 alone	DNA Boost	Peptide Boost	PBS
IL-12 alone	0.00%	-	-	-
DNA Boost	96.90%	0.00%	-	-
Peptide Boost	99.98%	73.76%	0.00%	-
PBS	40.62%	97.37%	99.98%	0.00%

Figure 6.30 The effect of the peptide vaccine as a boost on IFN-γ producing splenocytes.

On day zero 400 μg of β_2mL21SESP with 25 μg of pUMVC3-mIL12 was injected IM into 10 C57BL/6 mice. On day 14 five of the mice received 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 administered IM, termed the DNA Boost. The other five receive 400 μg of pβ_2ML21SE and 25 μg of pUMVC3-mIL12 administered SQ, termed the Peptide Boost. There where 3 mice in the control groups IL-12 alone and PBS received only 25 μg of pUMVC3-mIL12 or PBS administered IM, respectively. A) Depicts graphically the average value for the ELISPOT as assay for each group and the average of duplicate ELISPOT assays for each mouse in the group. B) List the confidence levels, based on Student t-Test analysis of each group's ELISPOT data, at which one can say the values were statistically different.

Boost schedule

In this experiment, twenty-five 7-10 week old C57BL/6 (H-2K^b) mice (Charles River Laboratories) were divided into 3 test groups of 5 and 2 control groups of 5. The test groups were injected IM, into each hind leg, on day 0 with 400 μg of β_2mL21SESP with 25 μg of pUMVC3-mIL12 and then boosted with the same thing on either day 7, 14 or 28. The control groups received either 25 μg of pUMVC3-mIL12 or just PBS on days 0 and 14. For the test group spleens were isolated 14 days after the boost, corresponding to days 21, 28, and 42, respectively. One mouse from each control group was sacrificed on day 21, three from each group were sacrificed on day 28, and one from each group on day 42. Cells were counted and immediately assayed via an ELISPOT for the number of INF-γ producing splenocytes (Figure 6.31). The data shows that with over 95% confidence a boost 14 days after the primary immunization generates more IFN-γ producing viral-specific splenocytes at an average of 162 ± 148 spots/million splenocytes compared to when boost were given on day 7 (51 ± 17 spots/million splenocytes), or on day 28 (45 ± 10 spots/million splenocytes).

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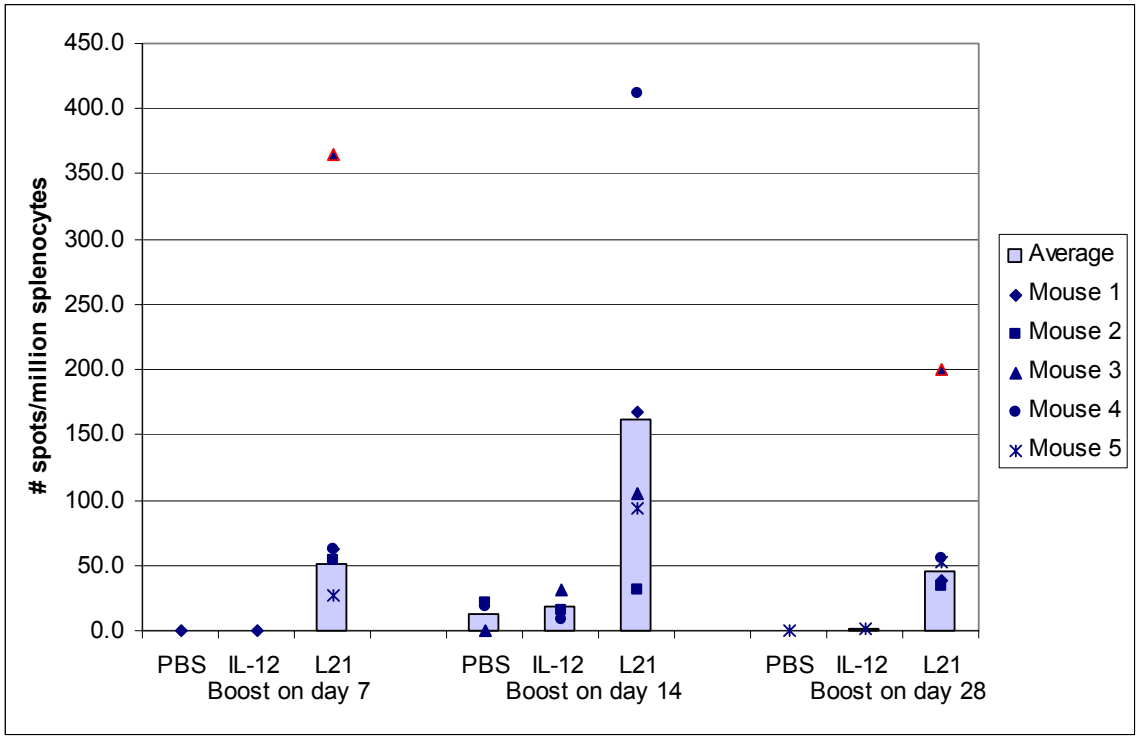


B

	L21 Boost Day 7	L21 Boost Day 14	L21 Boost Day 28
L21 Boost Day 7	0.00%	-	-
L21 Boost Day 14	95.25%	0.00%	-
L21 Boost Day 28	60.86%	96.26%	0.00%

Figure 6.31 The effect of boost time point on the number of IFN-γ producing splenocytes.

C57bBL/6 mice vaccinated on day 0 and boosted on either day 7, day 14, or day 28. L21 corresponds to five mice primed and boosted with 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12. IL-12 refers to mice primed and boosted only with 25 μg of pUMVC3-mIL12. PBS refers to mice primed and boosted only with PBS. For the IL-12 and PBS samples there was only 1 mouse on day 7 and 28 and 3 mice on day 14. The value of 365 spots/million splenocytes for mouse 3 L21 day 7 and the value of 200 spots/million splenocytes for mouse 3 L21 day 28, highlighted in red, was thrown out based on a standard Q test at 90% confidence. A) Depicts graphically the average value for the ELISPOT assay for each group and the average of duplicate ELISPOT assays for each mouse in the group. B) List the confidence levels, based on Student t-Test analysis of each group's ELISPOT data, at which one can say the values were statistically different.

STUDIES WITH LIVE SENDAI VIRUS

The next three studies were performed at the Trudeau Institute in Saranac Lake, New York under the guidance of Dr. David Woodland. A protection study was conducted to see if mice vaccinated with the DNA vaccine, β_2mL21SESP, would survive a lethal dose of Sendai virus. Tetramer staining was performed on days 4, 7, 10, and 14 to determine the relative amounts of viral specific CTLs in vaccinated and dummy vaccinated mice challenged with a sub-lethal dose. The viral titer in the lungs was determined on days 7 and 10 to determine if the vaccinated mice were clearing virus quicker than dummy vaccinated mice after a sub-lethal challenge. These studies were carried out concurrently with the dose and boost studies described above, which is why a 400 μg dose of β_2mL21SESP was used.

Protection study

This study was designed to determine if the vaccine could protect mice from a lethal challenge of Sendai virus. Thirty-five 10-11 week-old mice C57BL/6 (H-2K^b) mice (Jackson Laboratory) were divided into seven groups. Ten mice received injections of 400 μg of β_2mL21SESP IM divided equally into each hind leg on day 0 and again on day 14 and were classified as L21 DNA/ L21 DNA. Ten mice received 400 μg of β_2mL21SESP IM divided equally into the hind legs on day 0 and then 400 μg of pβ_2mL21SE SQ into the stomach area; these mice were called L21 DNA/ L21 Protein. Five mice received 400 μg of β_2mL21DMSP IM divided equally into the hind legs on day 0 and again on day 14 and were termed Dummy DNA/ Dummy DNA. Five mice received 400 μg of β_2mL21DMSP IM divided equally into the hind legs on day 0 and then 400 μg of pβ_2mL21SE SQ into the stomach area; these mice were referred to as

Dummy DNA/ L21 Protein. All injections in the first four groups included 25 μg of pUMVC3-mIL12. The final 5 mice were not injected with anything and thus were called Nothing.

On day 28, 14 days after the boost, mice were anesthetized with 250-300 μl of Avertin (Tribromoethanol, Appendix 1) administered intraperitoneally (IP) and were then administered what was believed to be a lethal dose of Sendai virus (2500 EID_{50}) intranasally. The survival of the mice was monitored for three weeks (Figure 6.32). Mice that looked dyspneic and adopted a hunched appearance at any time during the experiment were euthanized with 600 μl of Avertin.

It is obvious from the survival rate seen in the Nothing control that the chosen dose of 2500 EID_{50} was too small to be lethal. Although the difference in survival cannot be considered significant it is difficult not to notice that the only group in which all the mice survived was the Nothing group. In addition to the survival rates, mouse weight loss was monitored over the three week time period (Figure 6.33). Weight loss is generally an indicator that the animal's immune system is active and can provide a measure of the virus-induced morbidity (Ulmer, Donnelly et al. 1993). In immunized mice one would expect to see less weight loss and a more rapid return to their pre-infection weight as compared to controls. However, this was not observed in this experiment; as a whole, non-vaccinated mice showed less weight loss than the group of vaccinated mice as a whole.

A summary of peak weight loss given for each group is presented in Table 6.6. Weight loss in the L21 DNA/L21 DNA group is associated with dehydration when, on day 13, a water bottle was found broken and emptied in one of the cages, and was not included. When looking at the data in this fashion, there appears to be two distinct populations within each vaccinated group; one group showing significant weight loss

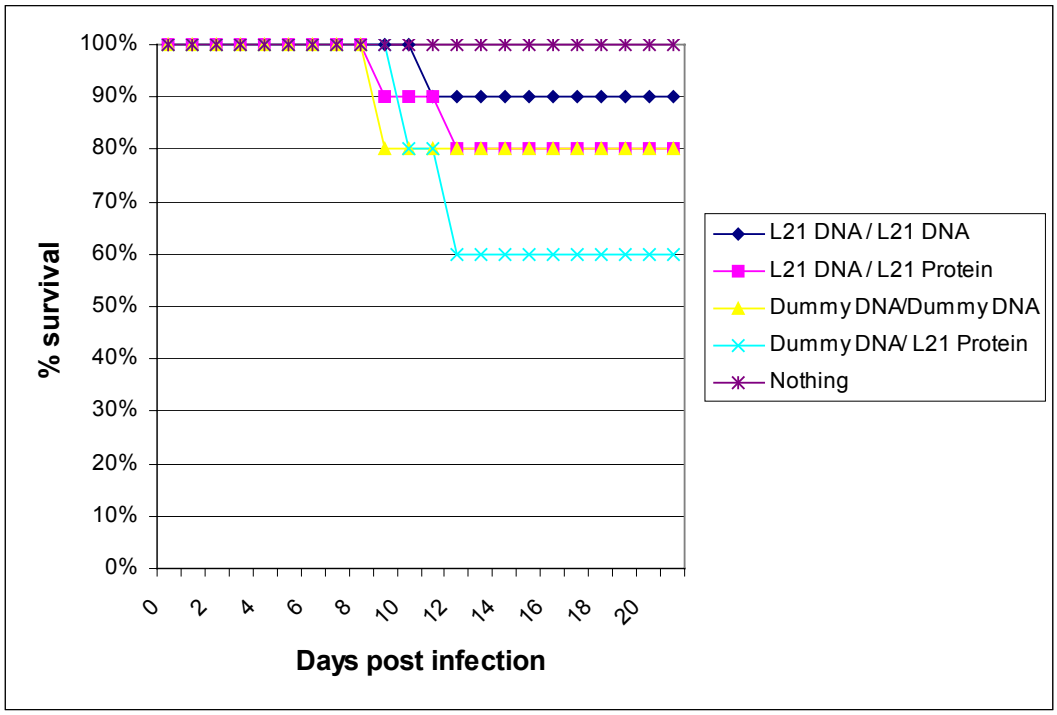


Figure 6.32 Results from protection study, percentage of mice that survived.

C57BL/6 were IM primed with 400 µg of β_2mL21SESP and 25 µg of pUMVC3-mIL12 or 400 µg of β_2mL21DMSP and 25 µg of pUMVC3-mIL12. On day 14 mice were boost IM 400 µg of β_2mL21SESP and 25 µg of pUMVC3-mIL12 or 400 µg of β_2mL21DMSP and 25 µg of pUMVC3-mIL12 or SQ with 400 µg of the recombinant protein β_2mL21SE and 25 µg of pUMVC3-mIL12. 14 days after the boost mice were given intranasally a 2500 EID_{50} dose of Sendai virus. Their protection from the virus was observed for 21 days. There were 10 mice in the L21 DNA/L21 DNA and the L21 DNA/ L21 Protein groups and only 5 mice in the other groups.

with the other group displaying moderate or no weight loses. For the L21 DNA/ L21 Protein, the moderate to no weight loss group accounts for 60% of the mice as compared to only 30% of the mice in the L21 DNA/ L21 DNA and 20% of the mice in both Dummy DNA/ Dummy DNA and Dummy DNA/ L21 Protein.

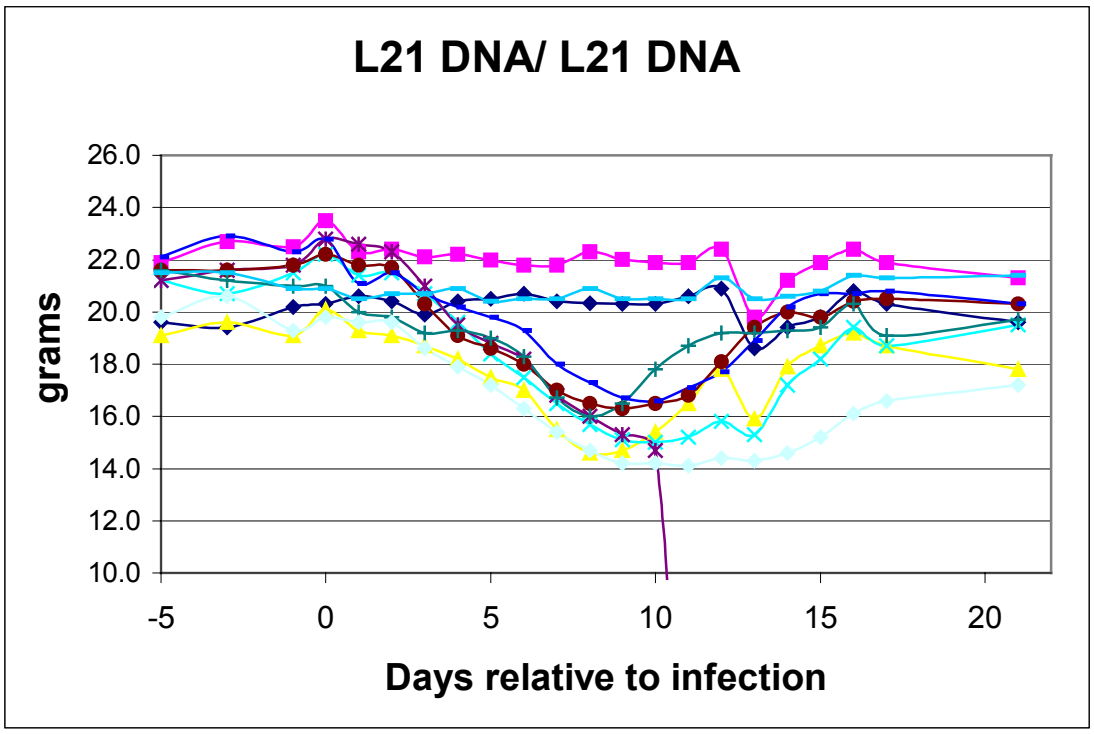
Grouped by % peak weight lose	L21 DNA/ L21 DNA	L21 DNA/ L21 Protein	Dummy DNA/ Dummy DNA	Dummy DNA/ L21 Protein	Nothing
0-5%	20%	30%	0%	0%	0%
5-10%	10%	20%	20%	20%	40%
10-15%	0%	10%	0%	0%	40%
15-20%	0%	0%	0%	0%	20%
20-25%	10%	0%	20%	0%	0%
25-30%	40%	20%	40%	40%	0%
over 30%	20%	20%	20%	40%	0%

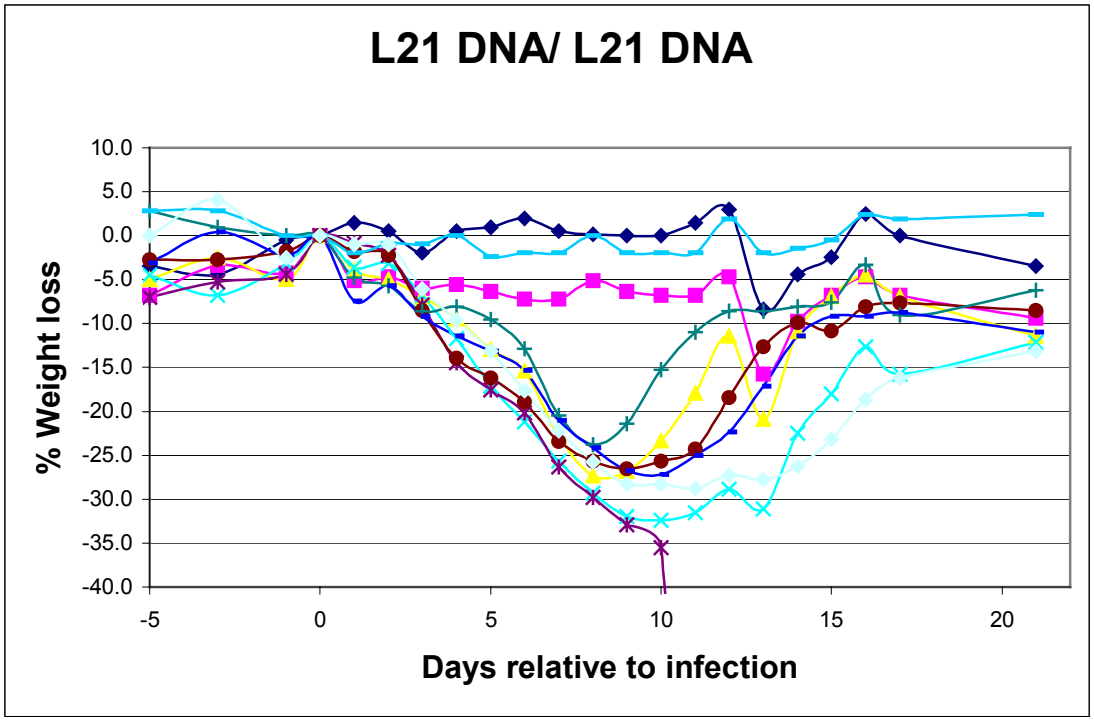
Table 6.6 Peak weight loss, grouped in 5% increments

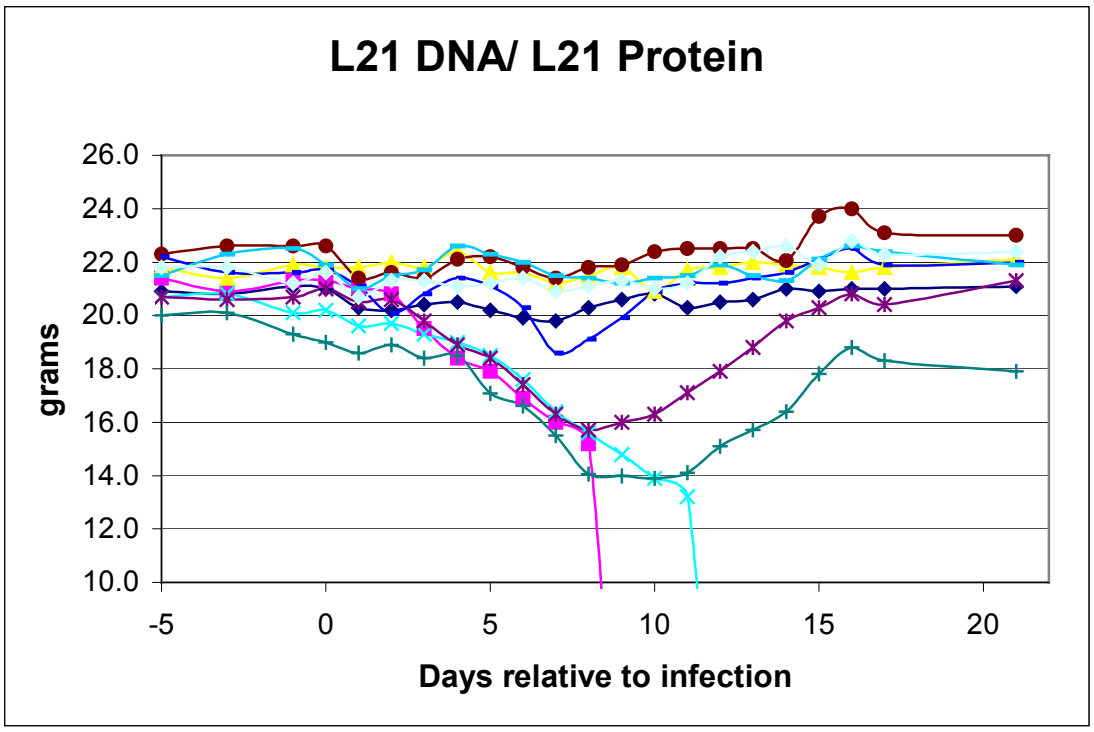
C57BL/6 were IM primed with 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 or 400 μg of β_2mL21DMSP and 25 μg of pUMVC3-mIL12. On day 14 mice were boost IM 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 or 400 μg of β_2mL21DMSP and 25 μg of pUMVC3-mIL12 or SQ with 400 μg of the recombinant protein β_2mL21SE and 25 μg of pUMVC3-mIL12. 14 days after the boost mice were given intranasally a 2500 EID_{50} dose of Sendai virus. Their weight loss was observed for 21 days. There were 10 mice in the L21 DNA/L21 DNA and the L21 DNA/ L21 Protein groups and only 5 mice in the other groups.

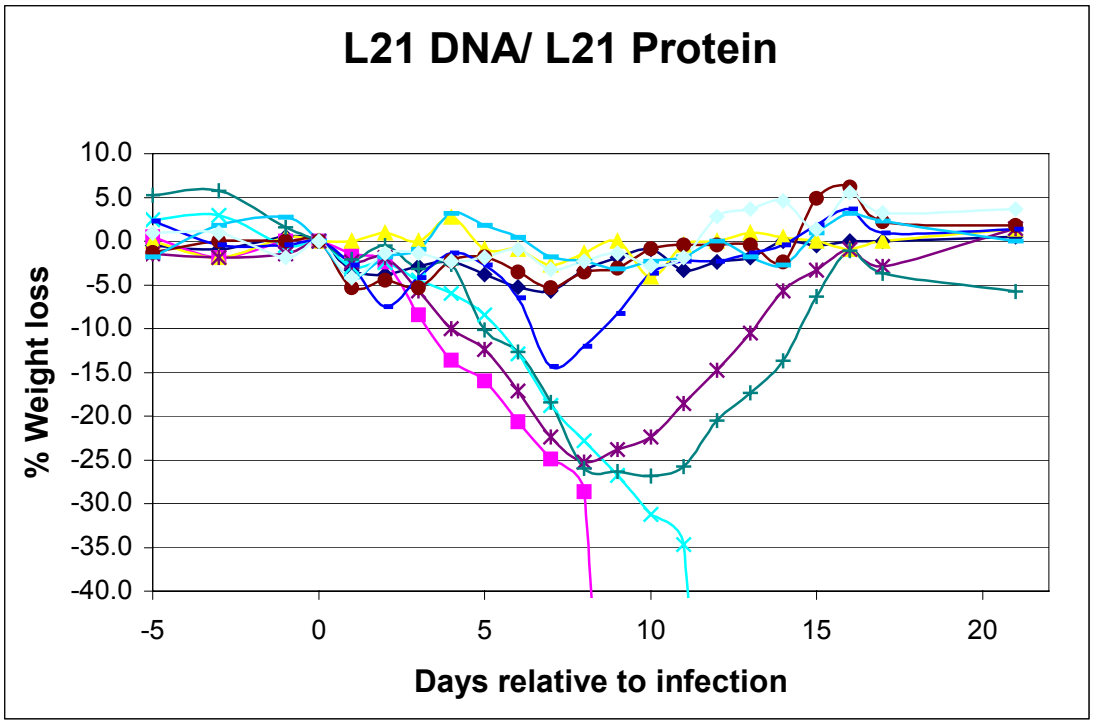
Figure 6.33 Mouse weight (grams) and % weight loss during protection study.

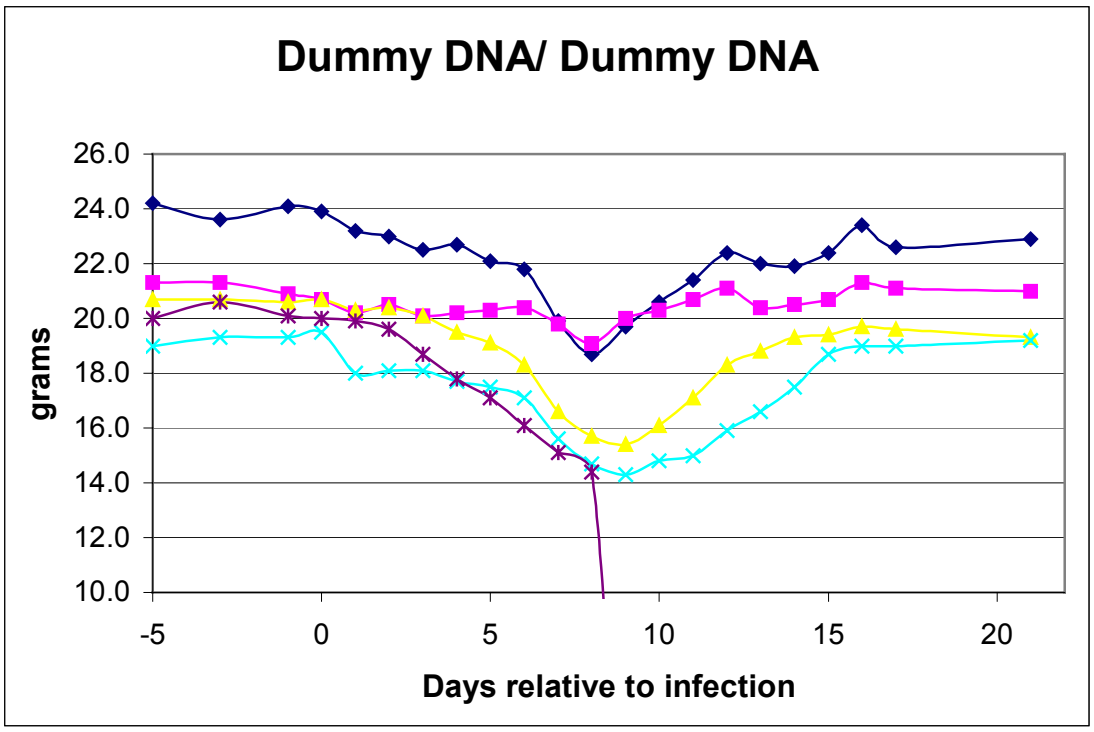
Experimental procedure described in directly above. (Below)

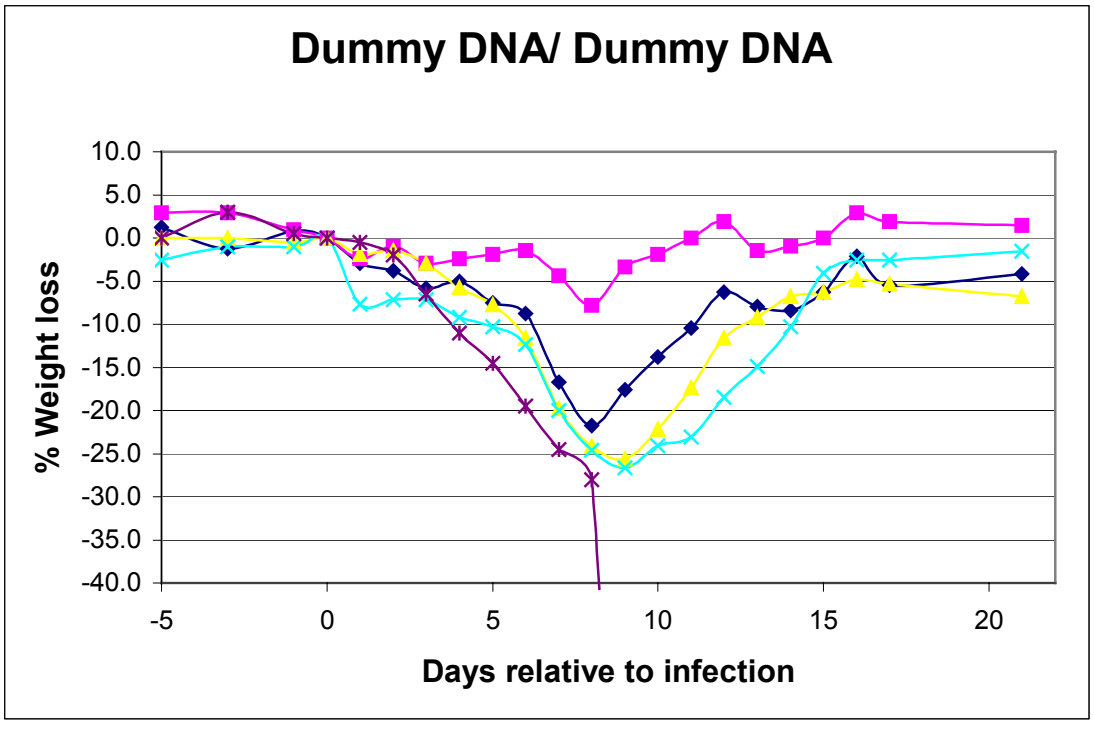

L21 DNA/ L21 DNA
grams
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
-5
0
5
10
15
20
Days relative to infection

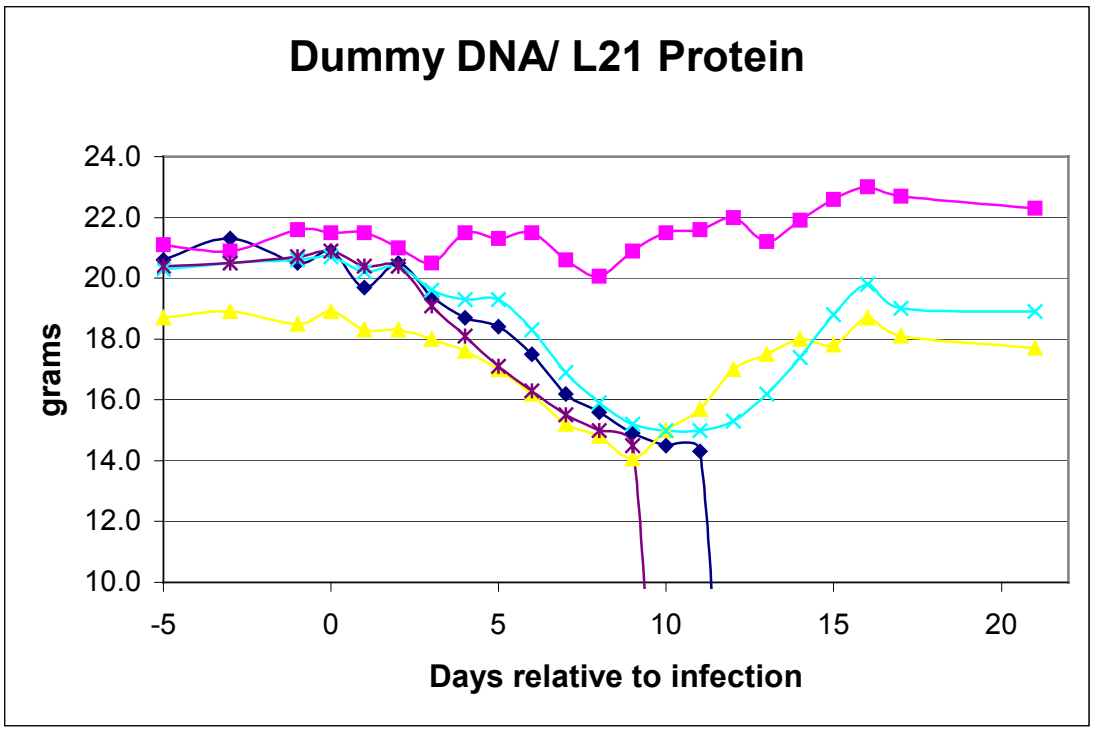

L21 DNA/ L21 DNA
% Weight loss
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
-40.0
-5
0
5
10
15
20
Days relative to infection

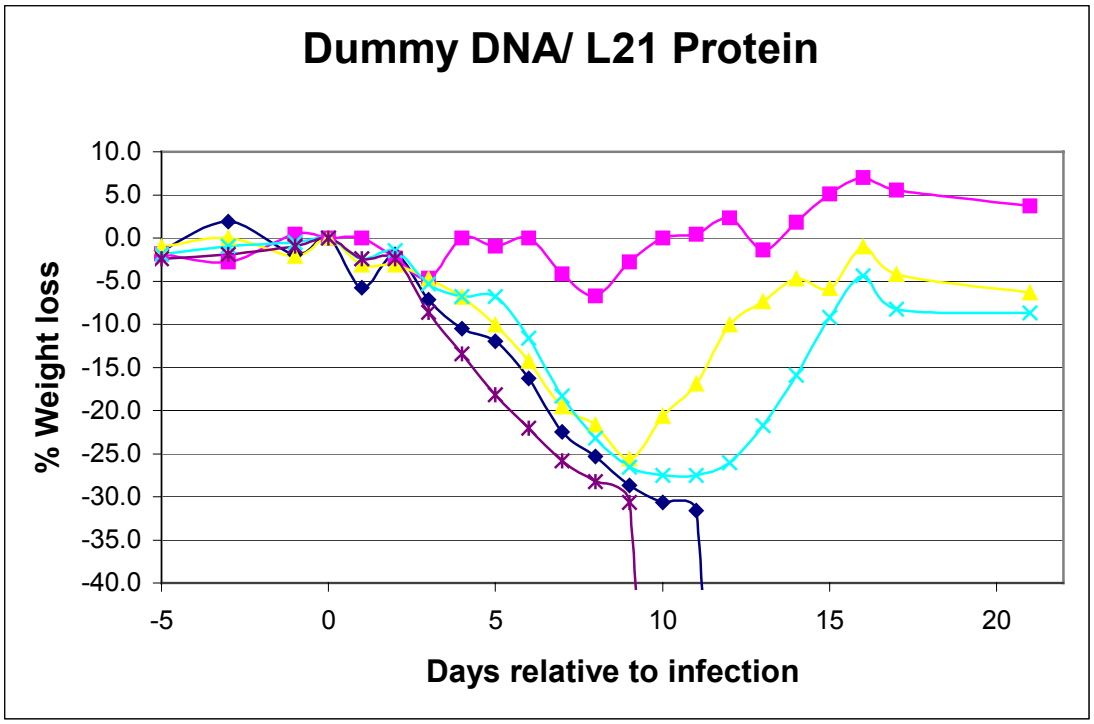

L21 DNA/ L21 Protein
grams
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
-5
0
5
10
15
20
Days relative to infection

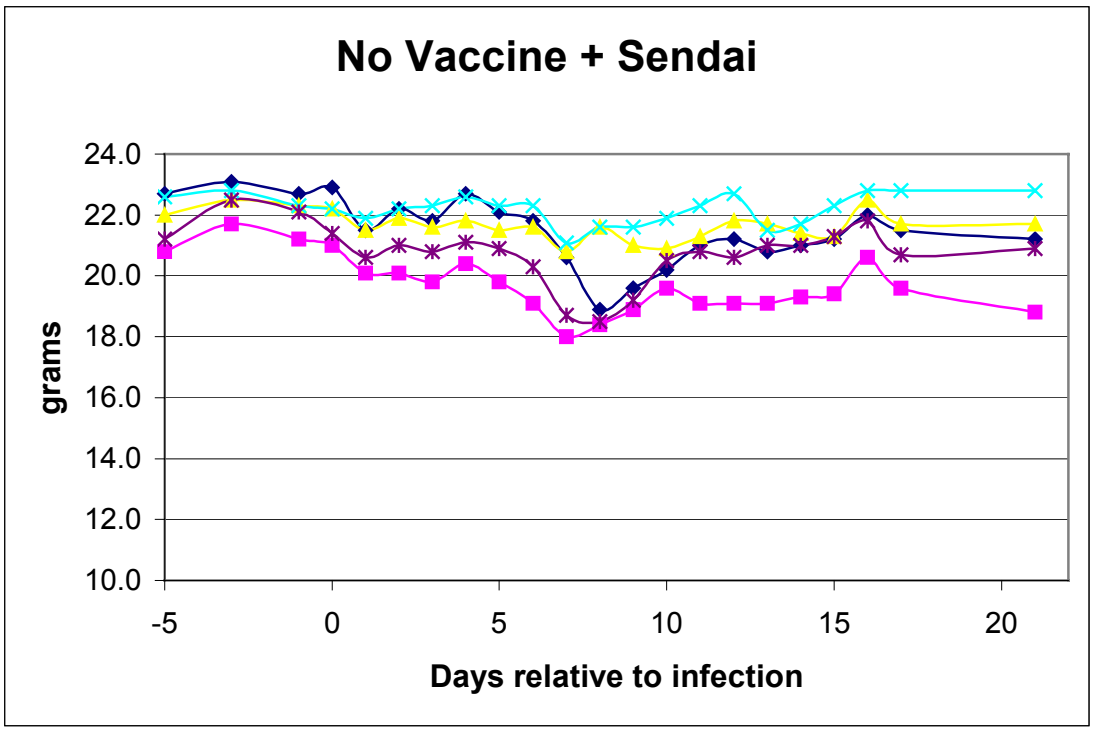

L21 DNA/ L21 Protein
% Weight loss
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
-40.0
-5
0
5
10
15
20
Days relative to infection

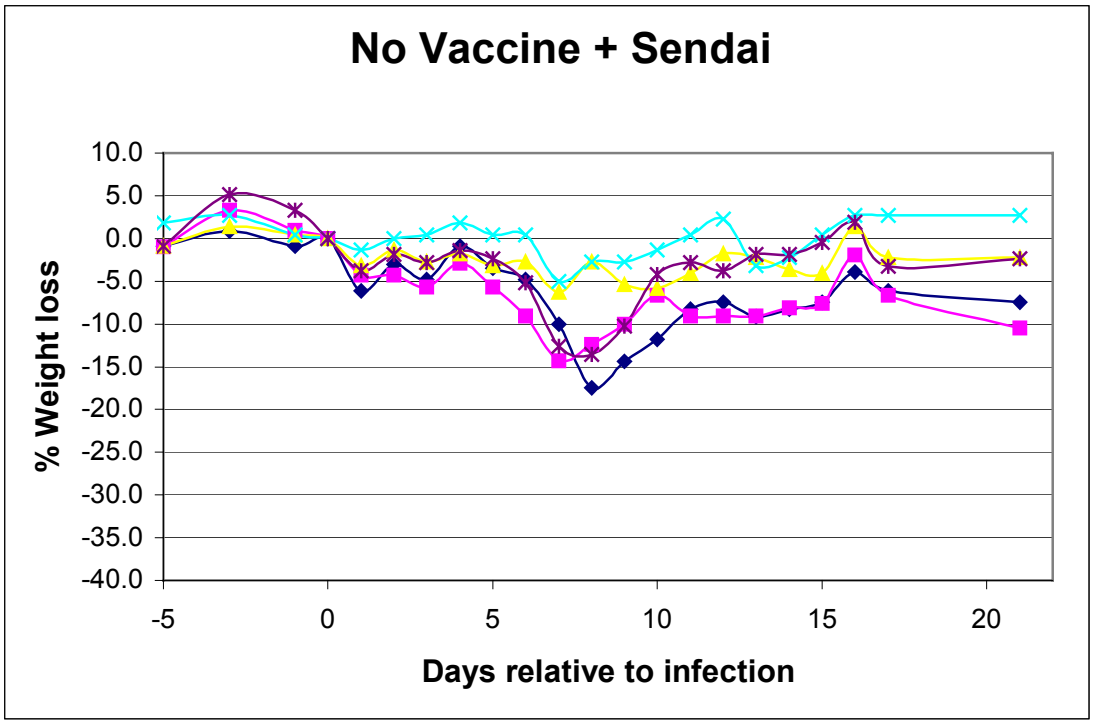

Dummy DNA/ Dummy DNA
26.0
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
grams
-5
0
5
10
15
20
Days relative to infection


Dummy DNA/ Dummy DNA
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
-40.0
% Weight loss
-5
0
5
10
15
20
Days relative to infection


Dummy DNA/ L21 Protein
grams
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
-5
0
5
10
15
20
Days relative to infection


Dummy DNA/ L21 Protein
% Weight loss
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
-40.0
-5
0
5
10
15
20
Days relative to infection


No Vaccine + Sendai
grams
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
-5
0
5
10
15
20
Days relative to infection


No Vaccine + Sendai
% Weight loss
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
-40.0
-5
0
5
10
15
20
Days relative to infection

Tetramer study

Twenty-four 9-10 week-old mice C57BL/6 (H-2K^b) mice (Jackson Laboratory) were divided into two equal groups. One group received 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 IM administered equally into the hind legs on day 0 and again on day 14. The other group IM received 400 μg of β_2mL21DMSP and 25 μg of pUMVC3-mIL12 divided equally into the hind legs on day 0 and again on day 14. On day 28, 14 days after the boost, mice were anesthetized with 250-300 μl of Avertin administered IP and then administered a sub-lethal dose of Sendai virus (125 EID_{50}) intranasally. Three mice in each group were sacrificed on days 4, 7, 10 and 14.

The bronchoalveolar lavage (BAL), spleen, lungs, and mediastinal lymph node (MLN) were collected. (Appendix 1) The mediastinal lymph node was the draining lymph node for the lung and the BAL was a sample collected by flushing the lungs. Red blood cells were lysed with Gey's solution. Lymphocytes were isolated from the BAL using a Percoll gradient where the cells of interest were found between the 40% and 80% layers. The B-cells and macrophages were removed from the spleens and MLNs by incubating the cells on a plate coated with goat anti-mouse IgG. Macrophages were also removed from the BAL samples by letting the macrophages adhere to a standard tissue culture plate. After the "panning" procedures non-adherent cells were collected and counted.

Cells from the spleen, lungs and MLN were analyzed separately, while the three BAL samples for each respective time point were pooled. Pooling of the BAL is necessary to obtain enough cells to assay. The cells from these collections were stained for viral-specific memory CD8$^+$ cells using a PerCP anti-mouse CD8a LY-2 antibody (BD Pharmingen), a FITC anti-mouse C44 antibody (BD Pharmigen) and a Sendai

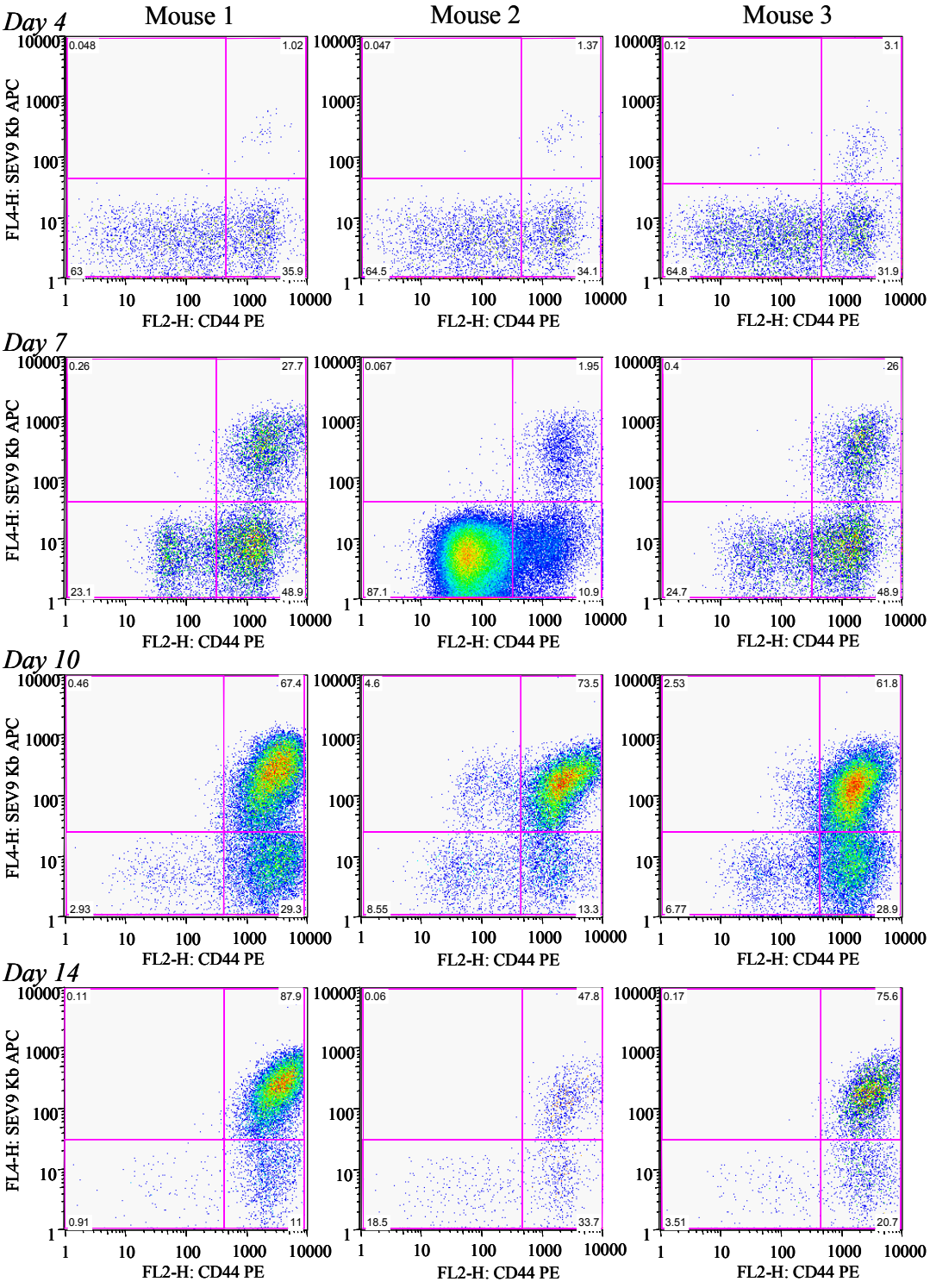
epitope specific PE H-2K^b MHC tetramer (Trudeau Institute) (Figures 6.29-6.33). The samples have been gated only to show CD8+ cells. The upper right hand quadrant represents high CD44 cells that were tetramer positive, (i.e. tetramer positive memory cells).

Notice by day 4, viral specific CD8$^+$ memory cells had arrived in the lungs of mice vaccinated with β_2mL21SESP, but not in mice injected with the dummy vaccine, β_2mL21DMSP. Since the Sendai virus is a respiratory infection, the early recruitment of memory cells to this area was noteworthy. Viral-specific CD8$^+$ memory cells do not appear in the lungs of dummy vaccinated mice until day 7. With almost 90% confidence one can say that the β_2mL21SESP vaccinated mice throughout the experiment consistently showed a higher percentage of viral-specific memory cells in the lungs.

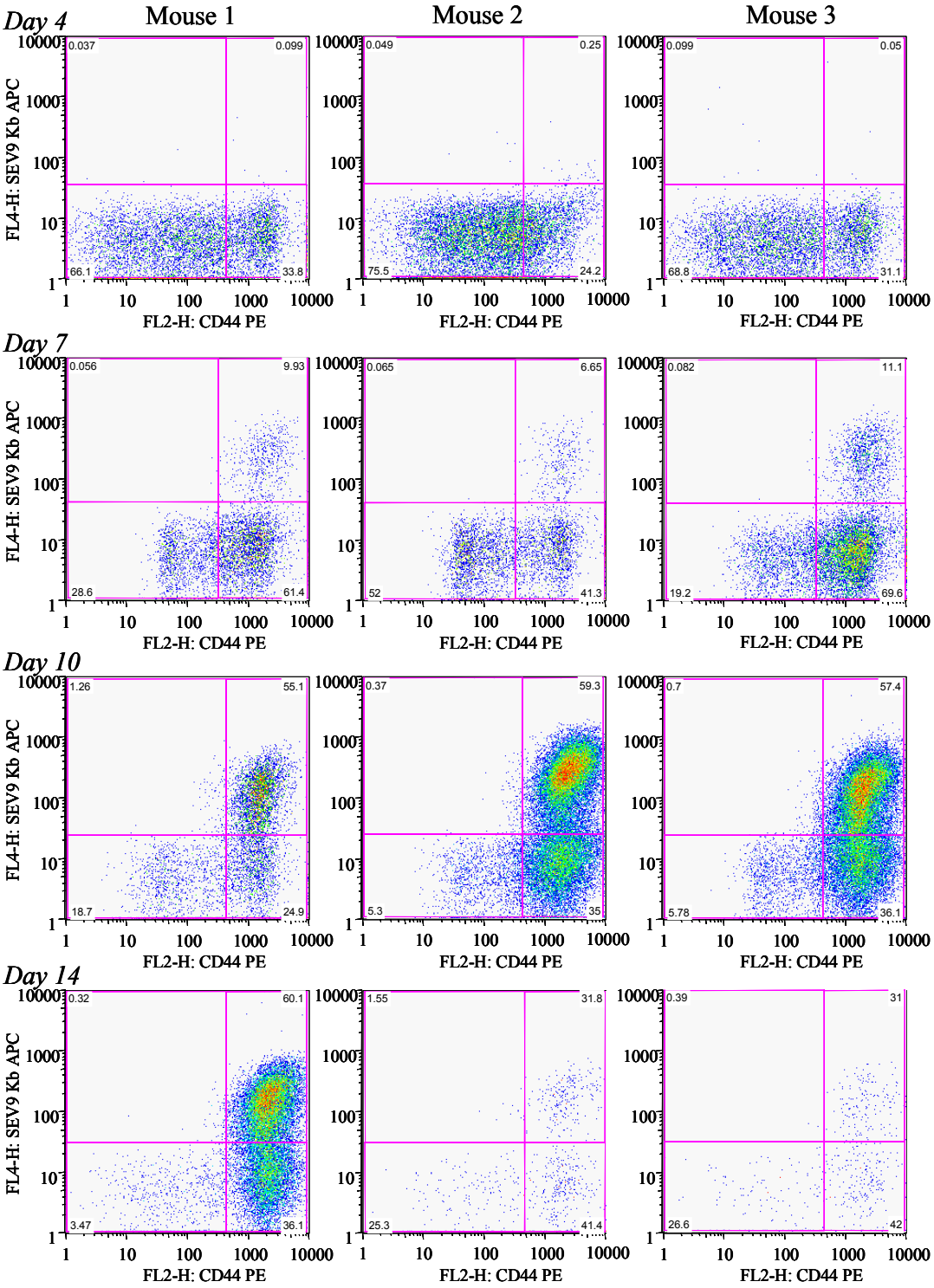
It was unfortunate that the BAL sample for day four was lost, because of the importance of this time point to show early arrival of viral-specific memory cells. Even still, relevant data can be obtained from the other time points. Notice that percentage of viral specific memory cells in the pooled BAL samples from β_2mL21SESP vaccinated mice were consistently higher than the BAL of dummy vaccinated mice.

The lack of viral-specific memory cells in the spleen and MLN on day 4 was expected because the infection was occurring in the lungs, thus initial T-cell will be recruited there. As time passes increased percentages of viral-specific cells at these locations in protected mice would be expected. At almost a 90% confidence level one state that take place in the MLN of β_2mL21SESP vaccinated mice. The same thing appears to be occurring in the spleen, but the confidence level that the numbers were distinct dropped below 90%. Unfortunately, with only three mice variability between them has a large effect on the ability to find statistical relevance; even so the trend was that spleens from β_2mL21SESP had higher percentages of viral-specific memory cells.

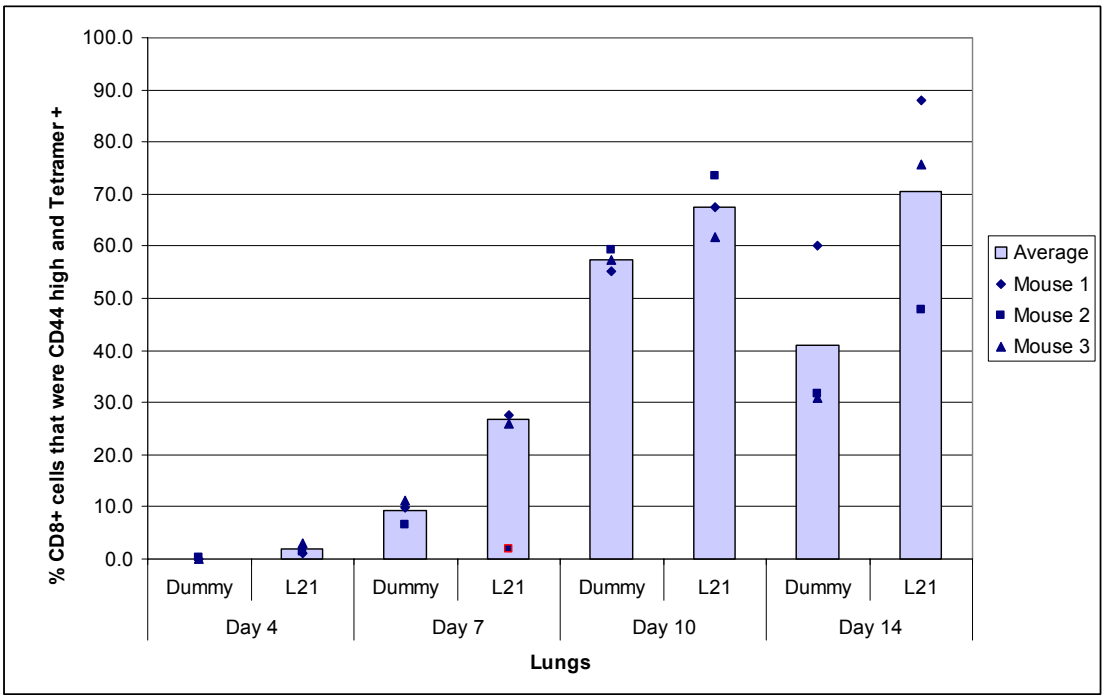
A Tetramer stains for viral specific memory $CD8^+$ cells in the lungs 0f β_2mL21SESP Mice



B Tetramer stains for viral specific memory $CD8^+$ cells in the lungs of dummy vaccinated mice



C Average % $CD8^+$ cells that were CD44 high and tetramer positive



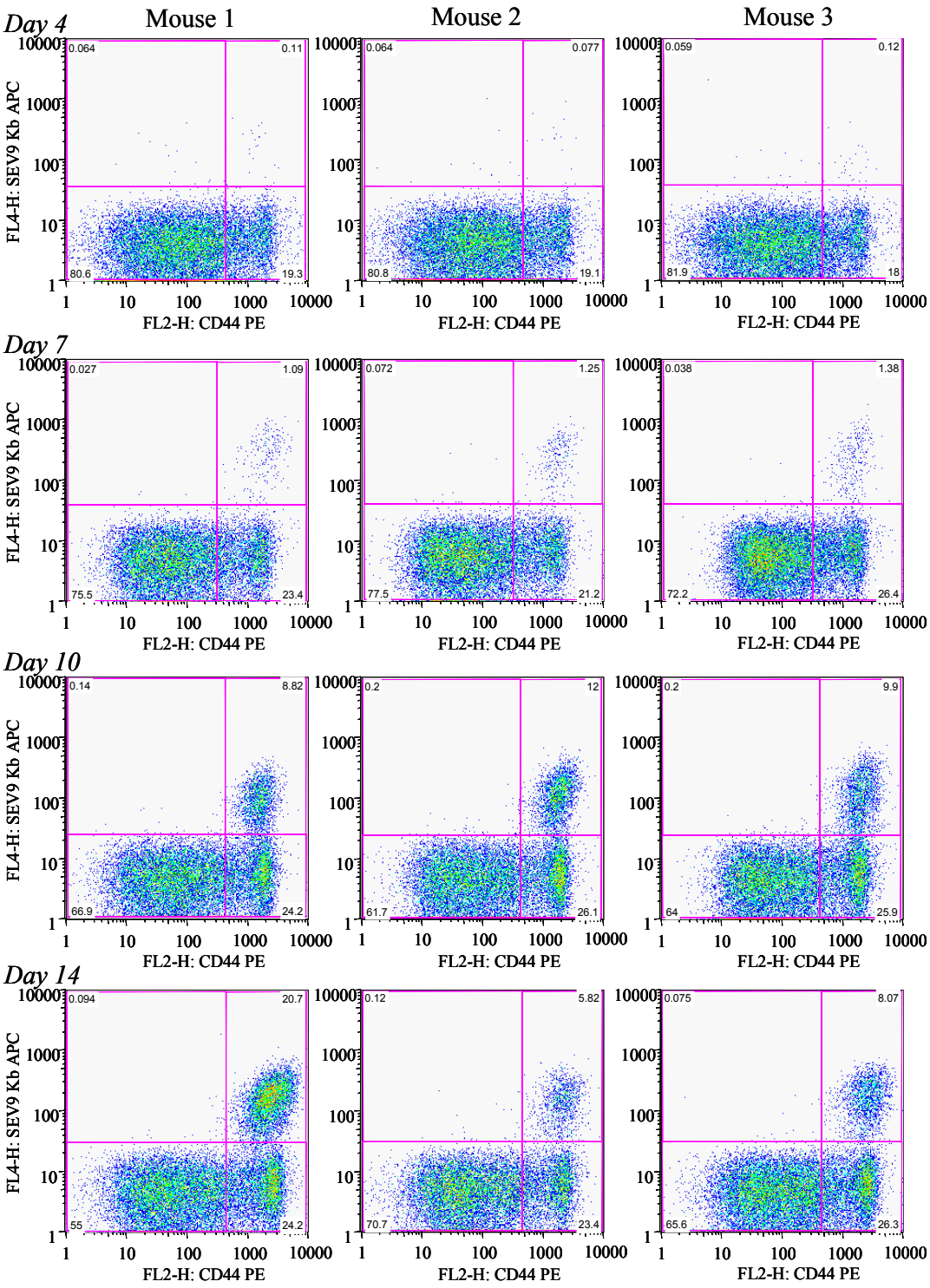
D

	Dummy Day 4	Dummy Day 7	Dummy Day 10	Dummy Day 14
L21 Day 4	94.17%	-	-	-
L21 Day 7	-	99.76%	-	-
L21 Day 10	-	-	95.45%	-
L21 Day 14	-	-	-	87.47%

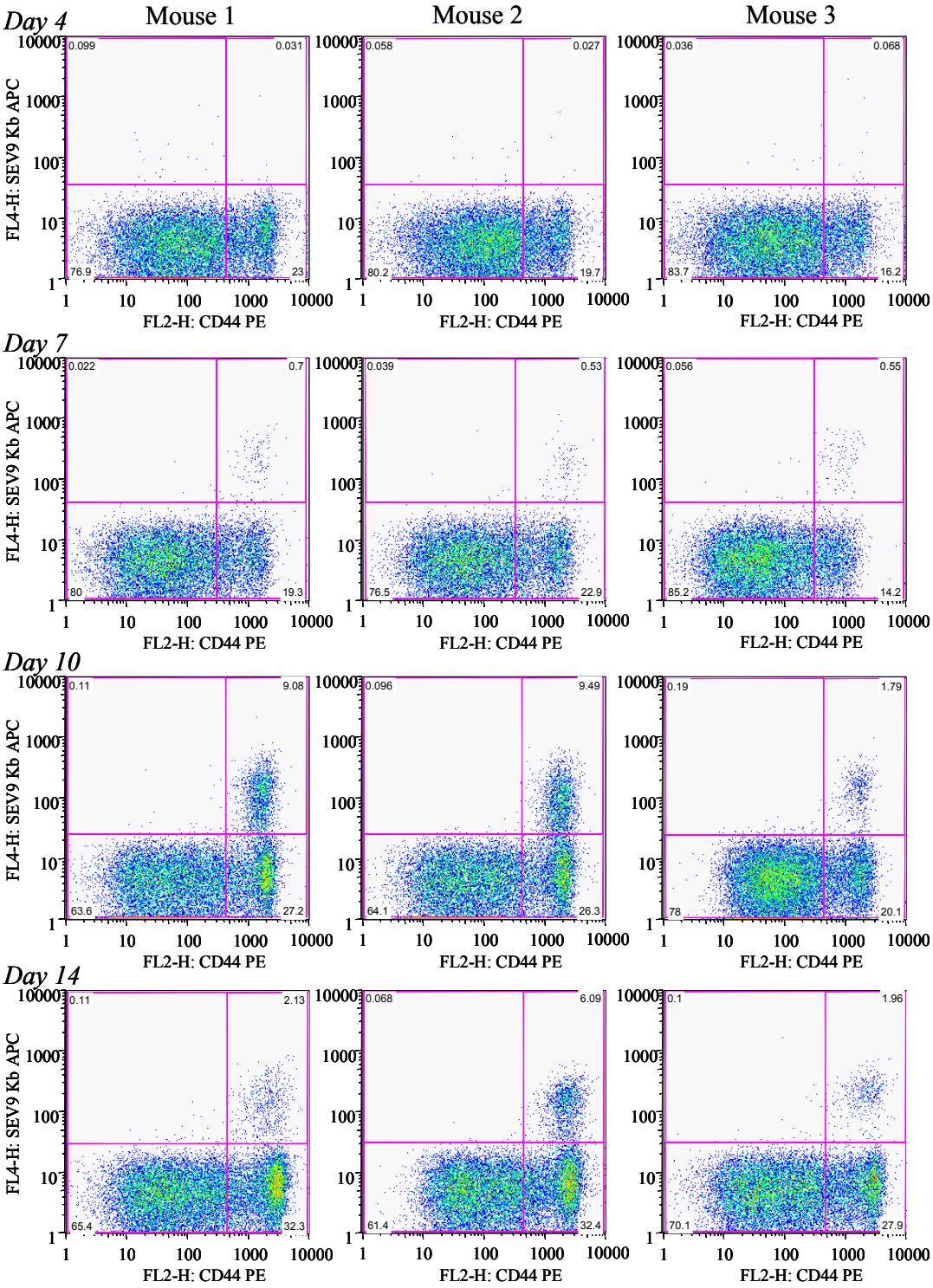
Figure 6.34 Tetramer staining for viral specific memory CTL in the lungs.

C57BL/6 mice were infected intranasally with 125 EID_{50} Sendai virus 14 days after the boost and sacrificed 4, 7, 10, and 14 days post. They were stained with anti-CD8, anti-CD44 and the Sendai epitope tetramer, SEV9 Kb. The data is $CD8^+$ gated with CD44 on the x-axis and SEV9 Kb on the y-axis. Each group displays 3 mice per day. The average response for L21 Day 7 only includes 2 mice because of the unusually high number of $CD8^+$ cell in the sample highlighted in red. A) The staining results for mice vaccinated and boosted, 14 days later, with 400 µg β_2mL21SESP and 25 µg of pUMVC3-mIL12. B) The staining results for mice vaccinate and boosted, 14 days later, with 400 µg β_2mL21DMSP and 25 µg of pUMVC3-mIL12. C) The percentage of $CD8^+$ cells that were CD44 high and recognized the tetramer. D) Student t-Test analysis for the level of confidence that the values were statistically distinct.

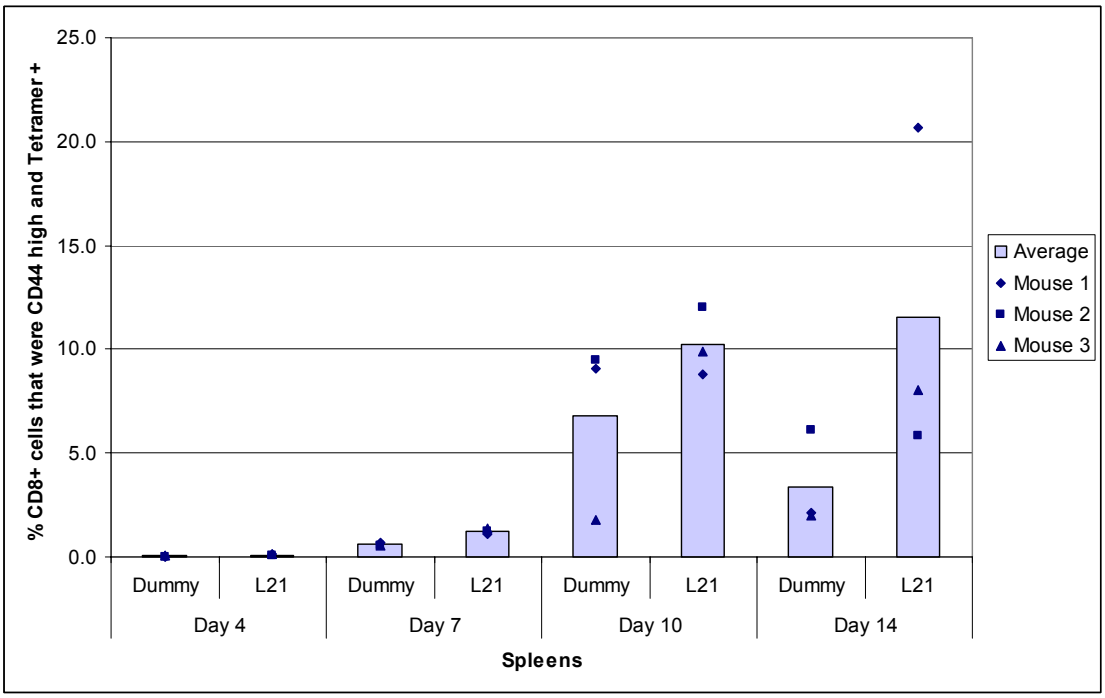
A Tetramer stains for viral specific memory $CD8^+$ cells in the spleens of β_2mL21SESP Mice



B Tetramer stains for viral specific memory $CD8^+$ cells in the spleens of Dummy Vaccinated Mice



C Average % $CD8^+$ cells that were CD44 high and tetramer positive



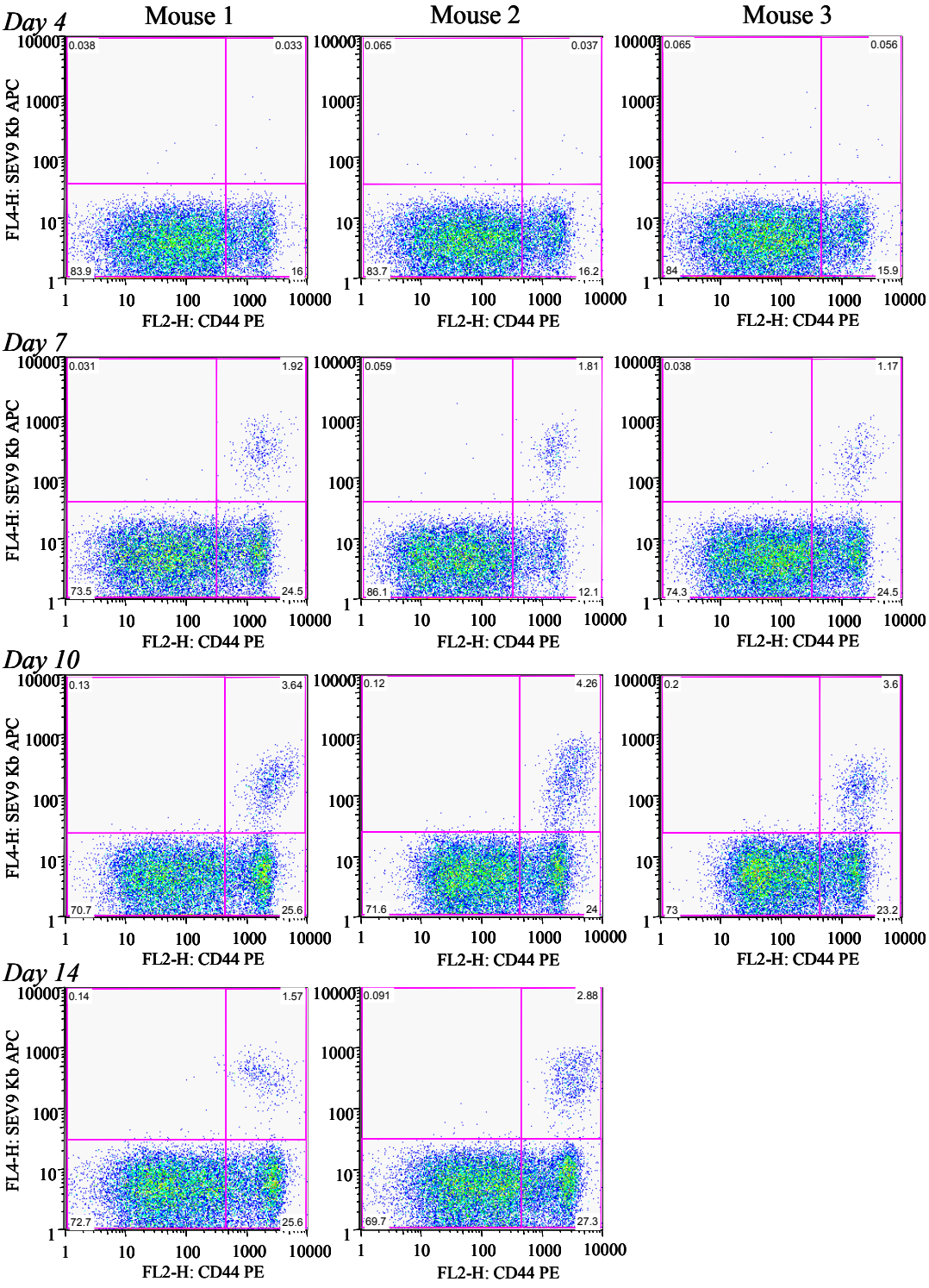
D

	Dummy Day 4	Dummy Day 7	Dummy Day 10	Dummy Day 14
L21 Day 4	96.94%	-	-	-
L21 Day 7	-	99.71%	-	-
L21 Day 10	-	-	73.45%	-
L21 Day 14	-	-	-	83.31%

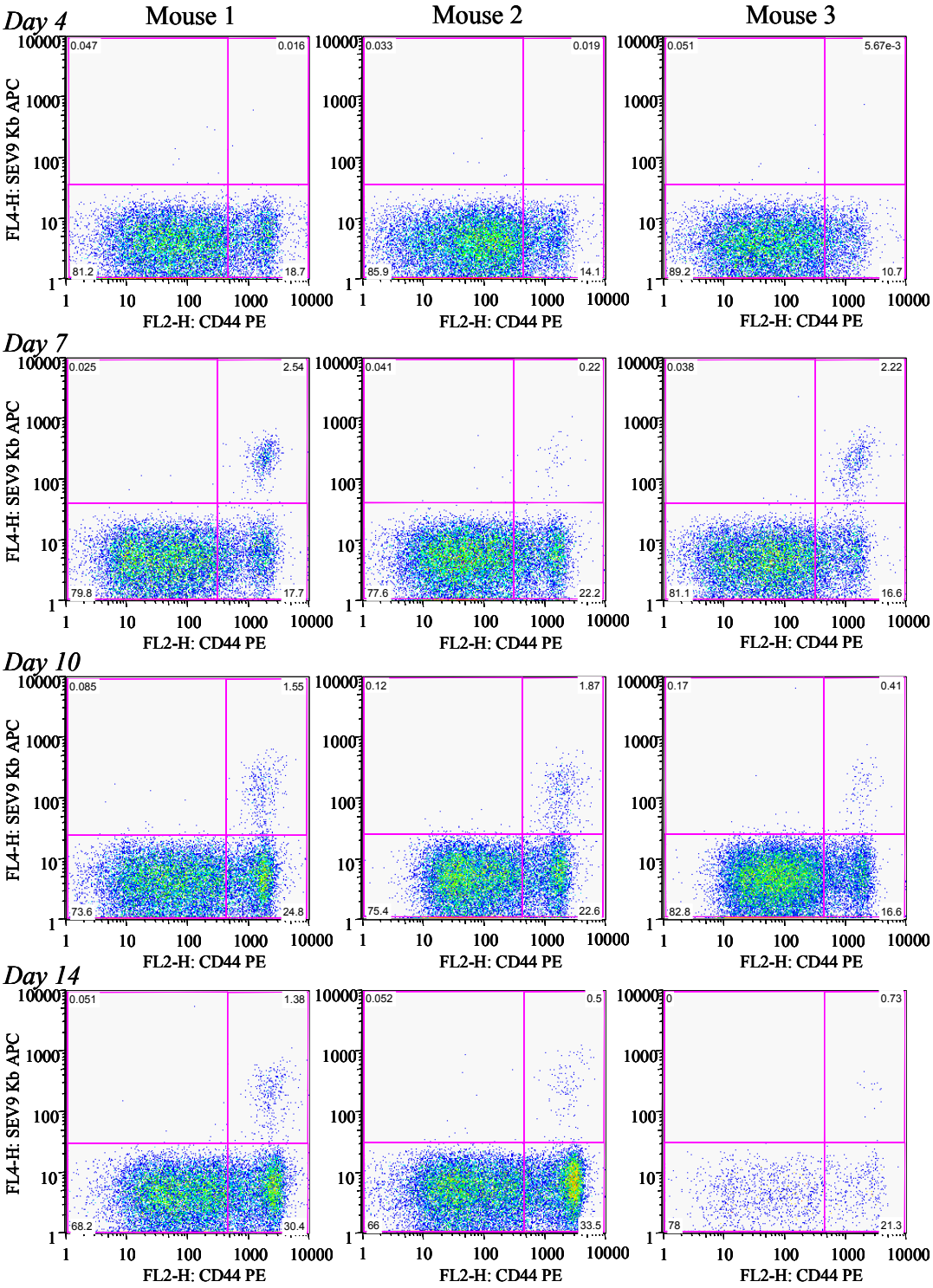
Figure 6.35 Tetramer staining for viral specific memory CTL from the spleen.

C57BL/6 mice were infected intranasally with 125 EID_{50} Sendai virus 14 days after the boost and sacrificed 4, 7, 10, and 14 days post. They were stained with anti-CD8, anti-CD44 and the Sendai epitope tetramer, SEV9 Kb. The data is $CD8^+$ gated with CD44 on the x-axis and SEV9 Kb on the y-axis. Each group displays 3 mice per day. A) The staining results for mice vaccinated and boosted, 14 days later, with 400 μg β_2mL21SESP and 25 μg of pUMVC3-mIL12. B) The staining results for mice vaccinate and boosted, 14 days later, with 400 μg β_2mL21DMSP and 25 μg of pUMVC3-mIL12. C) The percentage of $CD8^+$ cells that were CD44 high and recognized the tetramer. D) Student t-Test analysis for the level of confidence that the values were statistically distinct.

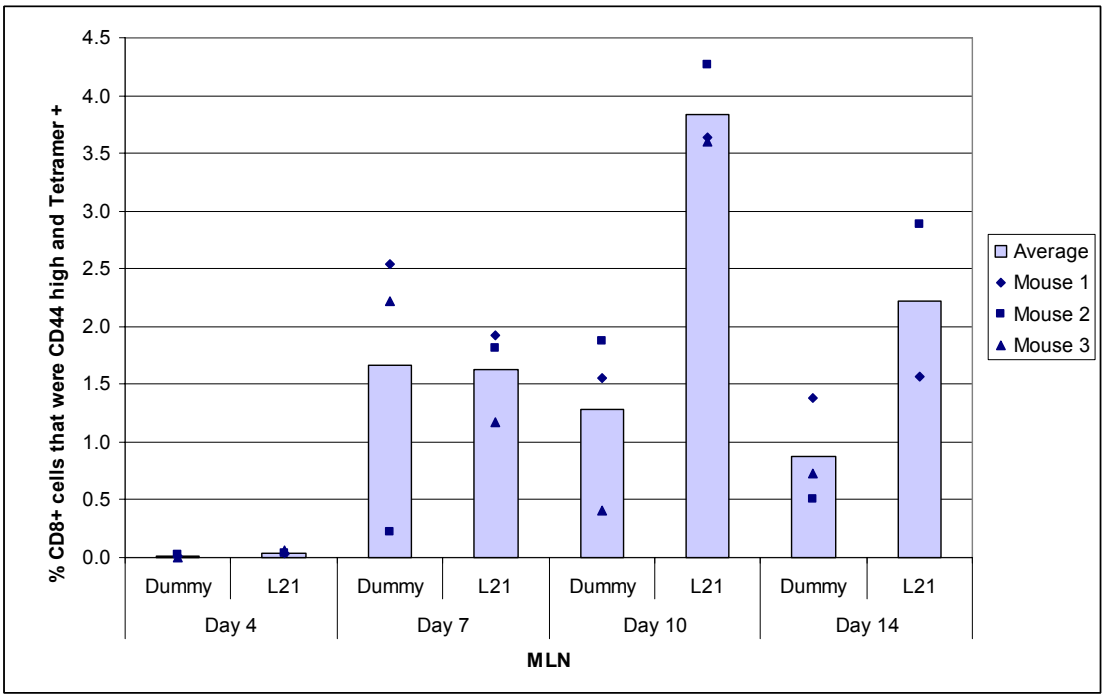
A Tetramer stains for viral specific memory $CD8^+$ cells in the MLN of β_2mL21SESP Mice



B Tetramer stains for viral specific memory $CD8^+$ cells in the MLN of Dummy Vaccinated Mice



C Average % CD8+ cells that were CD44 high and tetramer positive



D

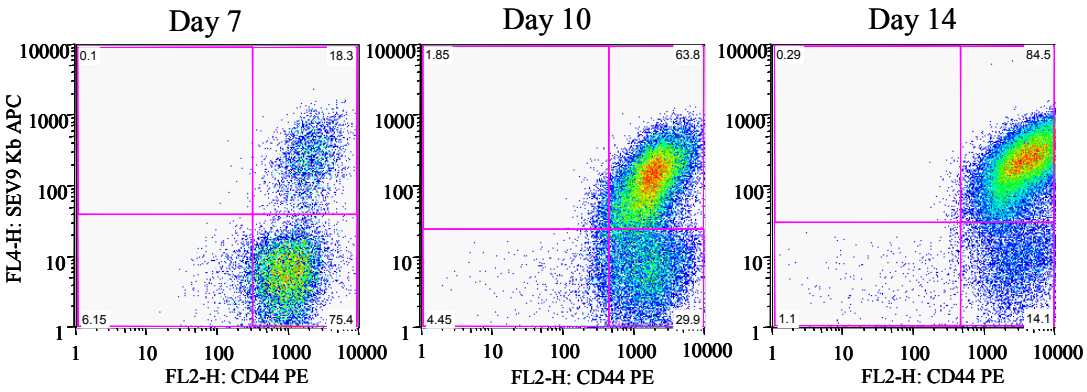
	Dummy Day 4	Dummy Day 7	Dummy Day 10	Dummy Day 14
L21 Day 4	97.48%	-	-	-
L21 Day 7	-	2.62%	-	-
L21 Day 10	-	-	99.35%	-
L21 Day 14	-	-	-	89.28%

Figure 6.36 Tetramer staining for viral specific memory CTL from the Mediastinal lymph node (MLN).

C57BL/6 mice were infected intranasally with 125 EID_{50} Sendai virus 14 days after the boost and sacrificed 4, 7, 10, and 14 days post. They were stained with anti-CD8, anti-CD44 and the Sendai epitope tetramer, SEV9 Kb. The data is $CD8^+$ gated with CD44 on the x-axis and SEV9 Kb on the y-axis. Each group displays 3 mice per day, except for the day 14 L21 when a sample was lost. A) The staining results for mice vaccinated and boosted, 14 days later, with 400 µg β_2mL21SESP and 25 µg of pUMVC3-mIL12. B) The staining results for mice vaccinated and boosted, 14 days later, with 400 µg β_2mL21DMSP and 25 µg of pUMVC3-mIL12. C) The percentage of $CD8^+$ cells that were CD44 high and recognized the tetramer. D) Student t-Test analysis for the level of confidence that the values were statistically distinct.

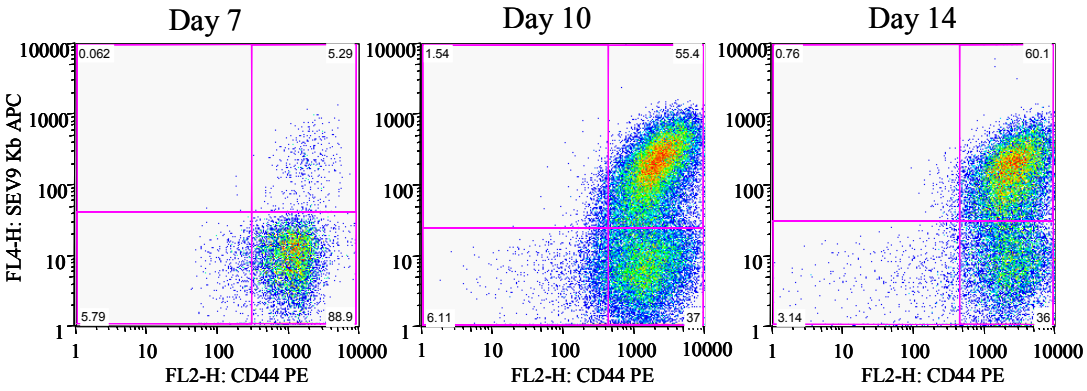
A

Tetramer stains for viral specific memory $CD8^+$ cells in pooled BAL samples from β_2mL21SESP Mice



B

Tetramer stains for viral specific memory $CD8^+$ cells in pooled BAL samples from Dummy Vaccinated Mice



C

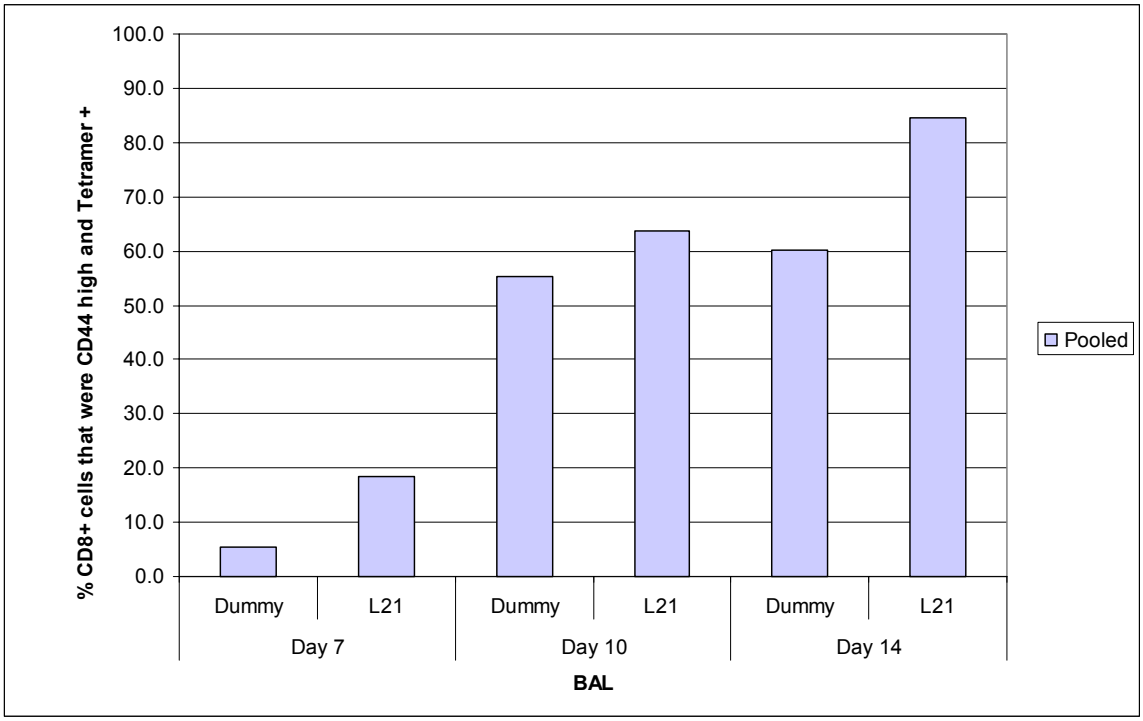


Figure 6.37 Tetramer staining for viral specific memory CTL from the pooled bronchoalveolar lavage (BAL).

C57BL/6 mice were infected intranasally with 125 EID_{50} Sendai virus 14 days after the boost and sacrificed 4, 7, 10, and 14 days post. They were stained with anti-CD8, anti-CD44 and the Sendai epitope tetramer, SEV9 Kb. The data is $CD8^+$ gated with CD44 on the x-axis and SEV9 Kb on the y-axis. A) The staining results for mice vaccinated and boosted, 14 days later, with 400 μg β_2mL21SESP and 25 μg of pUMVC3-mIL12. B) The staining results for mice vaccinated and boosted, 14 days later, with 400 μg β_2mL21DMSP and 25 μg of pUMVC3-mIL12. C) The percentage of $CD8^+$ cells that were CD44 high and recognized the tetramer. Each group displays the pooled BAL from 3 mice per day. The sample for day 4 spilled, so there are no results. Also, since these are pooled results no Student t-Test was performed.

Lung viral load study

Twenty-four 9-10 week-old mice C57BL/6 (H-2K^b) mice (Jackson Laboratory) were divided into two equal groups. One group received 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 injected IM divided equally into the hind legs on day 0 and again on day 14. The other group received 400 μg of β_2mL21DMSP and 25 μg of pUMVC3-mIL12 injected IM divided equally into the hind legs on day 0 and again on day 14. On day 28, 14 days after the boost, mice were anesthetized with 250-300 μl of Avidin administered intraperitoneally and then administered a sub-lethal dose of Sendai virus (125 EID_{50}) intranasally. Three mice in each group were sacrificed on days 4, 7, 10 and 14 and the lungs were removed. Lungs from mice sacrificed on days 7 and 10 were assayed for their viral load using an endpoint titration in embryonated hen eggs. (Appendix 1). Lungs from mice euthanized on days 4 and 14 were frozen, to be assayed later if deemed necessary. Figure 6.38 gives the results of the lung viral titers as the log of the EID_{50}. Unfortunately the number of mice studied per time point was only 3, which presents problems when looking for significance. With only 35% confidence, as calculated from using the Student t-Test, would one state that the log of the viral titer on Day 7 in L21 DNA/ L21 DNA vaccinated mice, 7.0 ± 1.7 log EID_{50}, was significantly lower than Dummy DNA/ Dummy DNA, 7.7 ±1.5 log EID_{50}. On day 10 with 88% confidence one could state that the L21 DNA / L21 DNA vaccinated mice, 0 ± 0 log EID_{50} had a lower viral titer than the Dummy DNA/ Dummy DNA vaccinated mice, 1.3 ± 1.2 log EID_{50}.

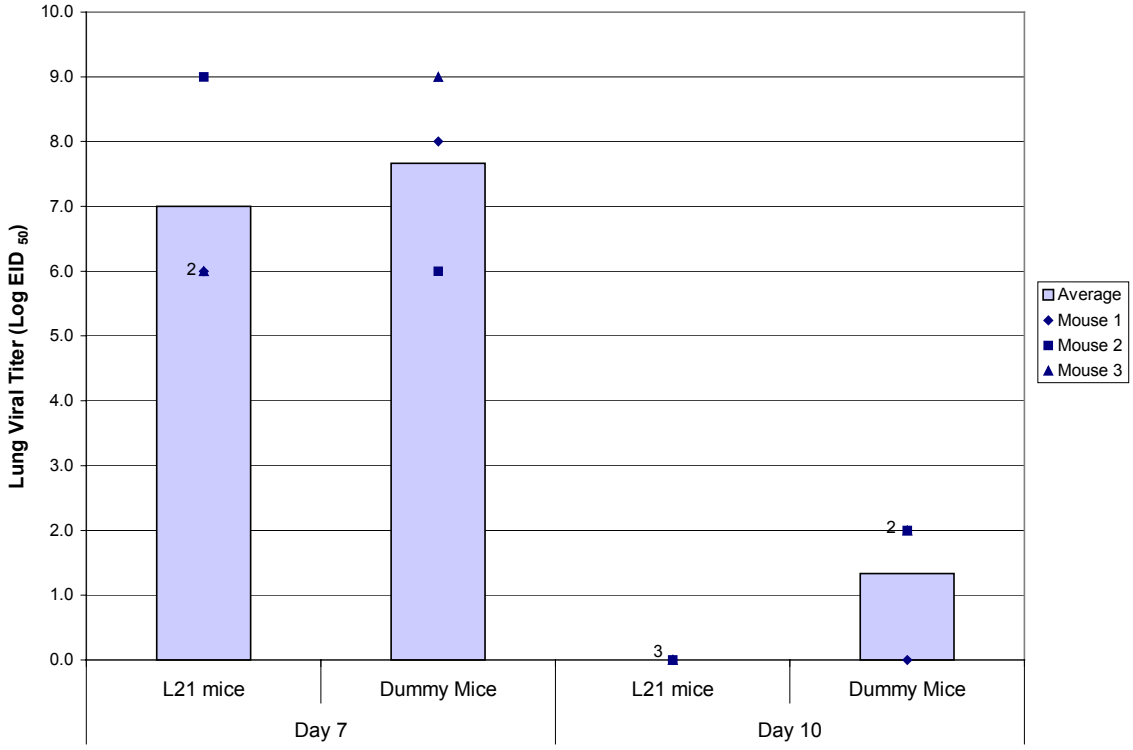


Figure 6.38 Lung viral titer data presented as the log EID_{50}.

C57BL/6 mice were IM primed and boosted 14 days later with 400 μg of β_2mL21SESP and 25 μg of pUMVC3-mIL12 or with 4 or with 400 μg of β_2mL21DMSP and 25 μg of pUMVC3-mIL12. 14 days after the boost mice were inoculated intranasally with 125 EID_{50} of the Sendai virus. Lungs were collected on day 4, 7, 10, and 14 post infection. Each group represents the average of 3 mice assayed in triplicate for the egg infectious dose.

DISCUSSION

An important component of vaccines against many viruses, but in particular against HIV, will be the development of a vaccine that is able to elicit a strong CTL response. (Pollack, Zhan et al. 1997; Rowland-Jones, Dong et al. 1999; Kaul, Rowland-Jones et al. 2001; Kaul, Rowland-Jones et al. 2001; Novitsky, Flores-Villanueva et al. 2001; Rowland-Jones, Pinheiro et al. 2001). A key step in CTL stimulation is the recognition of epitopes presented in the context of the MHC-I molecule. As a means to simulate this, the physical coupling of a viral epitope to the N-terminus of β_2m via a flexible linker has been explored. Others have examined this idea *in vitro*. Caras, in his dissertation in 1999, reported that a recombinant fusion protein with mouse β_2m linked via 21 AA to the H-2K^b Sendai NP immunodominant epitope could stimulate viral specific T-cell hybridomas (Caras 1999). Similarly, the Barber group at the University of Toronto, in 1998, showed that both cells pulsed with a recombinant fusion protein of human β_2m tethered via 12 AA to a H-2D^b-restricted immunodominant epitope from influenza virus NP and cells transfected with a plasmid encoding β_2m linked via 8 AA to a H-2K^d-restricted influenza NP epitope could function as target cells in a chromium release assay with splenocytes from influenza infected mice (Uger and Barber 1998). However, neither Caras nor Uger examined the effect linker size has on MHC-I binding or TCR recognition. But, more importantly, neither group assayed the ability of the β_2m-fusion vaccine to trigger an immune response *in vivo* or its ability to provide protection from a viral challenge. Both the effect of linker size and the vaccine's protective qualities as either a recombinant protein expressed in *E. coli* or as part of a mammalian expression vector are presented in this work.

β_2M-SENDAI EPITOPE VACCINE AS A RECOMBINANT PROTEIN

To look at the effect of linker size on the recombinant protein version of the β_2m viral fusion, β_2m was tethered to the H-2K^b Sendai NP immunodominant epitope using two different linker sizes: 10 AA and 15 AA. As a control, throughout the work, β_2m was tethered to a dummy epitope using a 15 AA linker. These constructs accompanied Caras' fusion protein, with the 21 AA linker, which allowed the examination of three different linker sizes. The proteins were engineered with extension PCR, cloned and expressed in *E. coli*, purified and then assayed *in vitro* and *in vivo*.

MHC-I upregulation

There were two aspects of the recombinant fusion protein vaccine that were important to establish before choosing which linker size to test *in vivo*. The first aspect was whether one of the linkers allowed the construct to bind the MHC heavy chain more efficiently. The second aspect was whether any of the linkers prevented the TCR from binding. An MHC-I upregulation experiment was performed to answer the first question and it was anticipated that the T-cell hybridoma stimulation experiment would answer the second.

Using the distance tool in Swiss PDB Viewer to examine the crystal structure of the H-2K^b molecule with the Sendai epitope (Fremont, Mutsamura et al. 1992), the length between the N-terminus of β_2m and the C-terminus of the epitope was estimated to be 20.6 Å. Linkers of 10 AA, 15 AA, and 21 AA were added on to the end of the N-terminus of β_2m using the build function of Swiss PDB Viewer. These linkers had an estimated size of 15.5 Å, 22.9 Å, and 32.2 Å, respectively. The linkers could have a

maximum length of 32.5 Å, 48.8 Å, and 68.3 Å respectively, if they adopted an extended beta sheet configuration. To provide a visual effect of the linker on the MHC-I molecule, using Swiss PDB Viewer, I modified the N-terminus of β_2m in the Fremont structure to include either a 10 AA linker or a 15 AA linker (Figure 7.1). Notice that the 10 AA linker in this configuration does not visually provide enough length to allow the fusion of the β_2m to the Sendai epitope, whereas the 15 AA linker provides the necessary length. Consistent with the modeling, the fusion proteins with the 15 AA and 21 AA linkers displayed a greater ability to stabilize the MHC-I molecule on the surface of the RMA-S cells than did the 10 AA linker (Figure 6.10). There was, however, no distinguishable difference between the 15 AA and 21 AA linkers, which would imply that the smaller 15 AA linker provided enough flexibility to allow the epitope to bind.

In 1999, Shields et al. showed that murine β_2m could stabilize the MHC-I molecule on the surface of RMA-S cells (Shields, Hodgson et al. 1999). The fact that the construct with a 10 AA linker showed any upregulation was slightly unexpected. Thus, one might be tempted to suggest that the stabilizing effect was due to the binding of β_2m and not the combination of the epitope with β_2m. If this were the case, then the same magnitude of stabilization would have been seen in the cells pulsed with the dummy epitope. However, these cells showed only a 2-fold increase stabilization of the MHC-I complex over the RMA-S control, whereas the cells pulsed with the recombinant β_2-microglobulin Sendai epitope fusion protein with a 10 AA linker showed a 7-fold increase.

The data from the pβ_2mL10SE studies are consistent with the published work of Uger et. al, 1998, which used a β_2m attached to a viral epitope with 12 AA acid linker (a maximum length of 39.0 Å) to pulse target cells in a chromium release assay (Uger and Barber 1998). Although the crystal structure for the MHC-I molecule with the allele and

epitope combination used by Uger has not been determined, the crystal structures of many other MHC-I molecules have been published. Table 7.1 summaries the minimal distance requirement for 25 known MHC-I molecules to attach the N-terminus of β_2m to

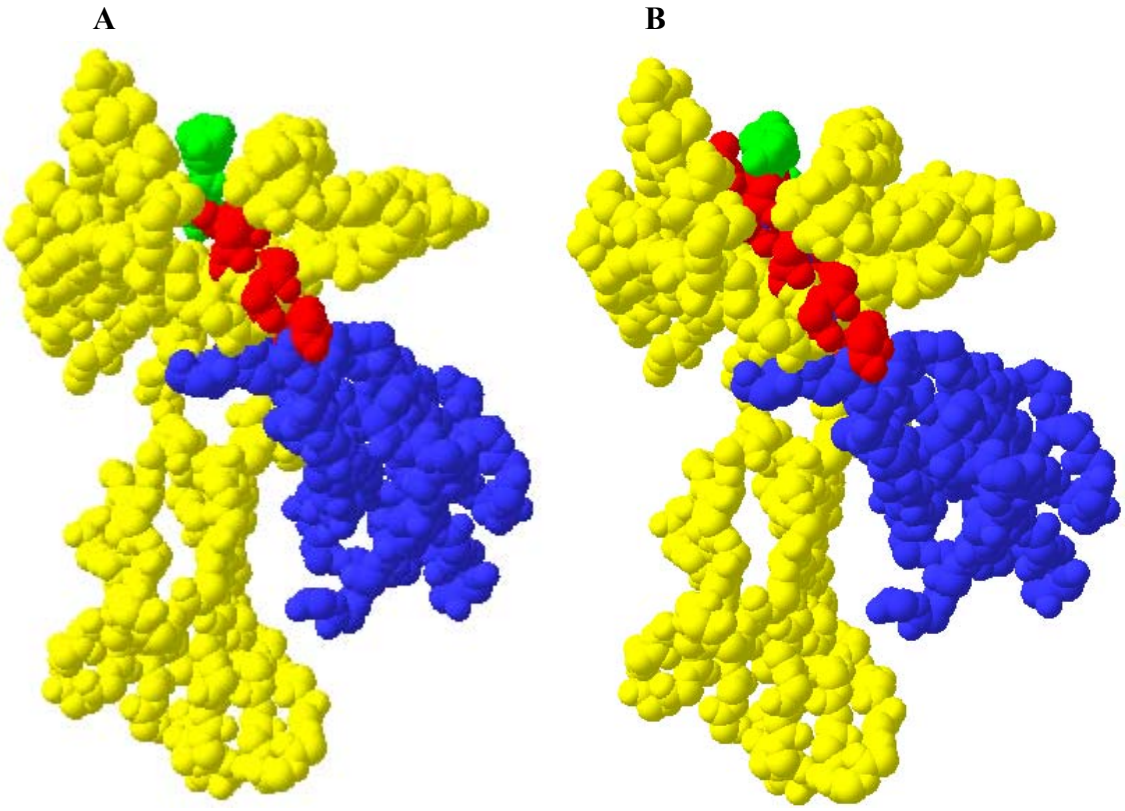


Figure 7.1 MHC-I structures modified to include the (Ser-Gly_4) repeat linker.

The original MHC-I structure was determined by Fremont. (Fremont, Mutsamura et al. 1992) A) The MHC-I structure with a 10 AA linker (red) attached to the N-terminus of β_2m (blue). B) The MHC-I structure with a 15 AA linker attached to the N-terminus of β_2m, with the Sendai epitope shown in green.

PBD ID	Donor	MHC Molecule	Epitope Length	Epitope Name	Minimal Linker Length in Å
1BZ9	Murine	H-2D^b	9	P1027	20.4
1FG2	Murine	H-2D^b	9	LCMV GP33	22.1
1CE6	Murine	H-2D^b	9	Sendai NP	18.7
1JPG	Murine	H-2D^b	9	LCMV NP396	20.3
1G7Q	Murine	H-2K^b	8	Mucin 1	21.0
1JPF	Murine	H-2K^b	11	LCMV GP276	21.0
1G7P	Murine	H-2K^b	9	Yeast aG	22.2
2VAB	Murine	H-2K^b	9	Sendai NP	20.6
1LEG	Murine	H-2K^b	8	Unknown	22.2
1NAN	Murine	H-2K^b	8	PBM1	20.1
1NAM	Murine	H-2K^b	8	VSV	20.4
1N59	Murine	H-2K^b	9	LCMV	22.3
1I7U	Human	HLA-A2	9	P1049-6v	20.4
1I7T	Human	HLA-A2	9	P1049-5V	21.2
1I7R	Human	HLA-A2	9	P1058	20.6
1IM3	Human	HLA-A2	9	HCMV US2	19.9
1DUZ	Human	HLA-A2	9	HCMV Tax	21.2
1JHT	Human	HLA-A2*0201	9	Mart-1	20.9
1JF1	Human	HLA-A2*0201	10	Mart-1	20.7
1I1F	Human	HLA-A2.1	9	HIV RT	21.5
1JGE	Human	HLA-B27	9	Unknown	22.5
IE28	Human	HLA-B5	8	HIV KM2	22.3
IE27	Human	HLA-B5	9	HIV KM1	19.8
1MI5	Human	HLA-B8	9	EBV	19.8
1KJV	Rat	Rtl-A1c	9	Unknown	19.5

Table 7.1 The distance from the N-terminus of β_2m to the C-terminus of the Epitope.

Using the Swiss PDB Viewer distance tool, with the N-terminus of β_2m as the first point and the C-terminus of the peptide as the second, the minimal distance needed to link β_2m to the viral epitope was estimated for 25 known MHC-I structures.

the C-terminus of the peptide while it sits in the MHC heavy chain groove. This distance was determined using the Swiss PDB Viewer distance tool with the first point being the N-terminus of β_2m and the second point the C-terminus of the peptide. Though the minimal distance varies within each allele based on the type of epitope presented, no distance observed was smaller than the length of the 12 AA linker used by Uger. Given that crystal structures are only snap shots of a dynamic interaction, the actual distance in a fluid system may be less, as the *in vitro* data for the shorter linker sizes suggests. Nevertheless, in the upregulation experiment, both the 15 AA and 21 AA linker constructs performed better than the 10 AA linker construct, thus providing evidence that the choice of linker size was important.

T-cell hybridoma stimulation assay

Unfortunately the T-cell hybridoma stimulation assay used to see if the T-cell receptor preferentially tolerated one of the linker sizes was not stringent enough to distinguish between the linkers or the dummy epitope control. The choice of linker size depended on the T-cell hybridoma clone that was used. The M1.1B3 clone preferentially tolerated the 21 AA linker and the M3.4D3 clone released more IL-2 in the presence of the 10 AA linker (Figure 6.16). The M2.5D10 cells were unable to respond to the constructs with any of the linker sizes (Figure 6.14). Note that it would be inappropriate to compare the amount of IL-2 released by one clone to that of another clone, because the maximum amount of IL-2 released is dependent on the clone as well as the clone's ability to recognize the peptide being presented (Cole, Hogg et al. 1995). The knowledge gained from this set of experiments was that when the recombinant fusion protein was presented

in the MHC-I complex, it would be able to stimulate some but not all TCR that recognized the peptide presented in a normal manner via the cytosolic pathway.

In vivo immune response determined with a 'traditional' and 'designed' experiment

Having established that all the β_2m-Sendai fusion proteins could stimulate some of the viral specific hybridomas *in vitro*, all three were tested *in vivo*. In two separate experiments, using two different injection methods, intramuscularly (IM) and subcutaneously (SQ), mice were vaccinated with the recombinant fusion proteins to determine which linker size would generate the greatest immune response. In both experiments the vaccinated mice failed to generate viral- specific cells that secreted IFN-γ in samples from either the spleen or Peyer's patches. The failure of the vaccines to stimulate the immune response *in vivo* was unexpected. As described in the Results section the use of the adjuvant, TiterMax Gold, might well explain the inability of the vaccines to generate a measurable cellular immune response during the first experiment. However, it was not used in the 'matrix designed' experiment, which showed similar negative results. At this point some might be tempted to discount the results from the 'designed' experiment because of a lack of familiarity with the technique. Although the technique has not gained popularity in academic settings, many pharmokinetic studies use similar approaches to determine the effect of drug combinations (Merle and Mentre 1995; Boza, De la Cruz et al. 2000; Siepmann, Kranz et al. 2000; Duffull, Mentre et al. 2001; Duffull, Retout et al. 2002). Furthermore, as discussed below, a 'designed' experiment identified the three major contributors for generating the production of IFN-γ secreting splenocytes with the DNA vaccine. Taking these observations into account led me to

believe that the recombinant fusion protein vaccine failed as a primary stimulator of the immune system.

The most likely explanation for the poor vaccination results would be the failure of the recombinant protein to exchange with β_2m *in vivo*. Our lab had previously shown that pβ_2mL21SE could exchange with β_2m on the surface of H-2K^b expressing cells *in vitro,* with a maximal exchange rate occurring at a concentration of 2 μg/ml (Caras 1999). However, these experiments were performed in a serum-free media without exogenous β_2m to compete for the binding site. Although I could not find the reported value for normal β_2m levels in mouse blood or lymph fluid, in humans the concentration of β_2m in the blood is approximately 2 μg/ml (Thakar, Rodrigues et al. 1992; Rodriguez, Cortes et al. 2000). A similar concentration likely exists in the mice and thus our vaccine would have to out-compete native exogenous β_2m. Therefore, before proceeding further with recombinant protein vaccine design, a better understanding of exchange rate in the presence of competing murine β_2m is necessary to predict the likelihood that the vaccine could exchange *in vivo*. Furthermore, because ultimately this vaccine would be used in human subjects, it would therefore be vital to understand the ability of the β_2m fusion protein to exchange on the surface of cells expressing human MHC-I molecules while in the presence of human β_2m. It has been reported that human β_2m has a higher affinity for both human and mouse MHC-I molecules than murine β_2m (Karlsson, Groth et al. 1980). Since the murine β_2m was used in the present work it may be prudent to switch to human β_2m for future studies with this type of vaccine construct.

β_2M-SENDAI EPITOPE VACCINE AS A MAMMALIAN EXPRESSION VECTOR

To look at the effect of linker size on the mammalian expression version of the β_2m viral fusion vaccine, β_2m was tethered to the H-2K^b Sendai NP immunodominant epitope using three different linkers of sizes: 10 AA, 15 AA, and 21 AA. Two additional controls were engineered to accompany the DNA vaccines, one control had a dummy (nonsense) epitope with a 21 AA linker and the other had only the 21 AA linker with no epitope at all. The constructs were engineered with extension PCR, ligated into the pVAX1 minimal mammalian expression vector, cloned in *E. coli*, purified and then assayed *in vitro* and *in vivo.*

T-cell hybridoma stimulation assay

Regrettably, little information was gained from the *in vitro* T-cell hybridoma stimulation assays on transfected cell lines, as exemplified by the data shown in Figure 6.24. The likely problem with these experiments was very low transfection efficiencies. The transfection efficiency into the RMA-S cells of a plasmid expressing the green fluorescence protein (GFP) was estimated at less than one in a thousand cells (data not presented). While the transfection efficiency into the NOR-10 cells was better, even then it was only between 6-9% for the GFP plasmid (data not presented). If it were determined to be desirable to repeat these experiments, then the construct should be cut out of the pVAX1 vector and ligated into an alternate vector, such as pCDNA3.1 (Invitrogen), that provides a mammalian selection marker. The selection marker would allow one to eliminate untransfected cells and generate a stable clone line. Uger et. al, 1998, employed a similar method to generate stably-transfected RMA-S cells that expressed their β_2m influenza epitope protein (Uger and Barber 1998).

For a few reasons the generation of stable clones was not conducted in this work. First, the T-cell hybridoma stimulation experiment with the recombinant proteins established that this assay would not be able to identify whether one of the linker sizes was optimal. Second, the pVAX1 vector meets the requirements established by the Food and Drug Administration for uses as a DNA vaccine in humans, whereas vectors such as pCDNA3.1 do not (FDA 1996,). Finally, since there are differences between the *in vitro* and *in vivo* environments, it was more prudent to test the DNA vaccines *in vivo*.

In vivo immune response determined with a 'traditional' and 'designed' experiment

Two *in vivo* experiments were conducted to establish the best linker sizes to use; one following a 'traditional' experimental design where several animals were given only one vaccine and assayed for the number of IFN-γ producing splenocytes. The other followed a 'matrix designed' experimental approach where animals where given multiple vaccines at various doses to determine the effect of any one vaccine. In both experiments the β_2m-Sendai epitope construct with the 21 AA linker performed best. As for the construct that performed second best, the data was inconsistent. The 10 AA linker out performed the 15 AA linker construct in the 'designed' experiment by a small margin of 40 spots/million splenocytes in the ELISPOT assay. In the traditional method, however, the 15 AA linker out performed the 10 AA linker construct by 174 spots/million splenocytes.

The $1/8^{th}$ 2 level factorial center cube design used in the 'designed' experiment was the minimum required to determine the effect of each variable assuming that each variable acted independently. To take into consideration all the possible interactions between the 6 variables a larger full 2 level factorial design with 64 experimental groups

would have been needed. Originally it was believed that the vaccine would act independently in such a fashion that increasing the dose of one of the vaccines would not affect the response of others. This may not have been the case, as is discussed later, when looking at the effect of high doses of the DNA vaccine. Therefore, the results from the 'traditional' method were probably a more accurate picture of the order of performance. Nevertheless the both the 'designed' experiment and the 'traditional' experiment collectively showed that the 21 AA linker outperformed the 10 AA and 15 AA linkers and that all three vaccines outperformed the controls. Also, the decreased response seen in the mice vaccinated with β_2mL10SESP further highlights the importance of choosing an appropriate linker size.

There might be some concern as to whether the protein expressed on the cell surface is in fact the β_2m-21 AA linker- Sendai epitope fusion protein or if it is only the Sendai epitope, after cleavage from the fusion protein by a cellular protease. Although not to be offered as a definitive answer, the observed difference between the vaccines with the 10 AA, 15 AA, and 21 AA linkers to stimulate viral specific splenocytes, as shown in Figure 6.26, suggests that unless the small changes in linker sizes have dramatic effects on the ability of cellular proteases to cleave the epitope, then one could assume that the fusion protein would be intact on the surface of the cell. The following experiments could provide additional support to the idea that *in vivo* an intact fusion protein was being surface expressed.

Unfortunately we do not have the tools to assess surface expression *in vivo*. As an alternative one could alter the β_2m molecule in a manner that does not allow it to associate with the MHC-I heavy chain. Point mutations in β_2m have been shown to decrease the ability of the molecule to associate with the MHC-I heavy chain (Shields, Hodgson et al. 1999). Presumably mutations could prevent the expressed fusion protein

from associating with the MHC heavy chain and becoming part of the MHC-I complex. If mice vaccinated with a plasmid containing β_2m point mutations were able to generate a viral specific immune response similar to mice vaccinated with the β_2mL21SESP vaccine, then this would provide evidence that the Sendai epitope was being cleaved. If mice were not able to generate the immune response, then one could deduce that the intact fusion protein was surface expressed.

A less direct method would be to remove the signal peptide from the vaccine design. Proteins expressed from this plasmid would be directed to the cytosol, thus exposing the protein to the standard protein processing via the cytosolic pathway. Presumably, mice vaccinated with this plasmid might generate a small viral specific immune response, but most likely one would see a noticeable reduction in the number of viral specific splenocytes when compared to the original vaccine.

As additional evidence that intact fusion protein was most likely being surface expressed one could look at the studies performed by Uger et al., 1998 (Uger and Barber 1998). They showed that if the surface peptides were stripped from cells transfected with their β_2m-influenza epitope, these peptides could not precondition potential target cells in a chromium release assay. Alternatively, peptides stripped from cells pulsed with the influenza epitope were able to condition cells as targets. From these experiments they concluded that the β_2m-influenza epitope was surface expressed. Unfortunately due to the low transfection efficiencies *in vitro* this would not be an option with our construct, when expressed by the pVAX1 vector. However, this specific issue of surface expression could potentially be addressed with an alternate vector such as pCDNA3.1

The failure of the plasmid vaccine to generate a mucosal response as measured by the number of IFN-γ producing cells in the Peyer's patches was probably due to the IM route chosen to deliver the vaccine and the choice of mucosal systems to analyze. The

IM injection is traditionally known for its ability to create a systemic immune response (Yokoyama, Hassett et al. 1997; Fu, Guan et al. 1999; Ledwith, Manam et al. 2000). For inducing a generalized mucosal immune response other routes such as oral or intranasal administration function better. Although IM DNA vaccines often fail to generate a widespread mucosal immune response they are known to produce a local mucosal immune response in the draining lymph node (Wolff, Malone et al. 1990; Bohm, Mertens et al. 1998). For the hind leg injection site used in these experiments the draining lymph node would be the popliteal node. The Peyer's patches were chosen instead because of their ability to offer data concerning a desired widespread mucosal response.

Dose response

The most fascinating observation presented in this work was the unusual dose response seen in the studies with the various amounts of β_2mL21SESP DNA vaccines and 25 μg of pUMVC3-mIL12. The data suggests that as the amount of a DNA vaccine, known to stimulate the production of IFN-γ producing splenocytes, was increased, the number of IFN-γ producing cells decreased. The doses of plasmid DNA used in this work were higher than those reported by others who have examined dose responses on DNA vaccinations (Bohm, Mertens et al. 1998; Fu, Guan et al. 1999; Johnson, Conway et al. 2000; Lee, Yoon et al. 2001). Fu et al., 1999, showed that as the amount of a DNA vector expressing the NP gene from influenza increased from 30 ng – 30 μg the number of CTL-specific splenocytes also increased (Fu, Guan et al. 1999). Bohm et al., 1998, Johnson et al., 2000, and Lee et al., 2001, showed that as the dose of their respective DNA vaccines increased to 100 μg the measured viral specific immune response also increased (Bohm, Mertens et al. 1998; Johnson, Conway et al. 2000; Lee, Yoon et al.

2001). To the best of my knowledge we have conducted the only known experiments with DNA vaccines up to 800 μg, with our starting dose of 100 μg as the highest previously reported value in a dose response study. These values have been reported as μg of plasmid and not moles of plasmid; thus one might want to suggest that molar values would have been more equivalent to the doses used in other works. On this note, since pVAX1 is a minimal mammalian expression vector, whereas the vectors used by the other groups were not, it would be likely that when comparing μg to μg, mice vaccinated in this work received high molar doses.

Our data suggests that doses over 200 μg begin to have a negative effect on the measured immune response with reductions occurring in an almost linear fashion (Figure 6.27). At first I was tempted to believe that this might be a general rule for all DNA vaccines. I began developing the idea that the effect of the increased dose was due to tissue damage that interfered with uptake of the vaccine. But I doubt that this was the case, because tissue damage results in the activation of the immune system, which would presumably benefit the development of the viral specific response (Goldsby, Kindt et al. 2000).

Another possibility was that with increased initial doses of DNA, the mice developed a specific immune response to the DNA itself. Thus when the boost was given the mouse was primed to prevent the uptake of the boost DNA. An interesting idea, but again I doubt this would be the case because I could not find published work demonstrating a specific immune response against naked DNA. Perhaps the increased DNA triggered an increased non-specific immune response. Outside of CpG motifs found in bacterial DNA being used as adjuvants, I could find no published work to confirm or disprove this idea. Since the DNA constructs in this work did not contain any known CpG motifs this type of effect was considered negligible.

If total DNA was an issue in defining a response it could easily be tested by supplementing the smaller doses of β_2mL21SESP with the pVAX1 vector, which would result in all doses having an equal amount of total DNA. If repeating the experiment in this fashion gave the same results, then there might be something to the idea that increased total DNA was a problem in DNA vaccinations. More work would then be needed to determine if the problem with total DNA was due to an idea I have proposed above or to a completely different reason altogether.

Perhaps the decrease in the generation of viral specific IFN-γ producing splenocytes with increasing doses of DNA is not a general feature of DNA vaccines, but instead is specific for this particular vaccine. There was a potential, due to the type of vaccine used, that the orientation of the viral epitope being displayed might be slightly different when presented by two different H-2K^b alleles on the surface of the same cell. A number of reports have shown that small variations in the peptide presentation on the same cell can cause partial or complete inhibition of T-cell activation (Sloan-Lancaster and Allen 1996; Preckel, Grimm et al. 1997; Stotz, Bolliger et al. 1999; Preckel, Hellwig et al. 2001). Thus one could imagine that, since the epitope was physically attached to a flexible linker, there exists the possibility that the epitope could be presented by different H-2K^b alleles in a similar but slightly different fashion, causing T-cell anergy. However, if this were the case one would expect this to occur regardless of the dose; and this was not what the data showed.

Another concern specific for our DNA vaccine has recently been exposed. For years the level of β_2m in the blood has been correlated with diseases such as HIV, rheumatoid arthritis, multiple myeloma and chronic myelogenous leukemia (Revillard, Vincent et al. 1982; Lifson, Hessol et al. 1992; Thakar, Rodrigues et al. 1992; Mocroft, Johnson et al. 1997; Rodriguez, Cortes et al. 2000; Moreau, Misbahi et al. 2002). In the

past it was believe that the high level of β_2m was a side effect of the disease and not a potential contributor to it. However, two very recent works, one by Bodnar et al., 2003, and the other by Xie et al., 2003, provide evidence that high levels of β_2m have negative effects on the activation of cytotoxic T-lymphocytes. Bodnar et al. have shown that a B cell line pulsed with 5 μM (60 μg/ml) β_2m showed significantly weaker CTL activation than cells that were not pulsed with β_2m (Bodnar, Bacso et al. 2003). Similarly, Xie et al. showed that in a dose-dependent manner concentration of β_2m greater than 10 μg/ml caused a down regulation of MHC-I and co-stimulatory molecules expressed on the surface of monocyte-derived dendritic cells (Xie, Wang et al. 2003). In the presence of β_2m these cells produced more of the immuno-suppressant cytokines: IL-6, IL-8, and IL-10 (Xie, Wang et al. 2003). Additionally, Xie et al showed that in the presence of β_2m the dendritic cells were less able to stimulate T-cells, as measured by lower IL-2 and IFN-γ release (Xie, Wang et al. 2003).

It has been established that the expression of naked DNA injected intramuscularly, as done in this experiment, is localized to the muscle it was injected into and the draining lymph node associated with that muscle (Wolff, Malone et al. 1990; Yokoyama, Hassett et al. 1997; Bohm, Mertens et al. 1998; Martin, Parker et al. 1999; Ledwith, Manam et al. 2000). Though, as mentioned earlier, no study has looked at doses in the range where this work showed a decreased response, it has been shown that expression levels of the encoded protein increase with increased DNA dosages (Fu, Guan et al. 1999). Therefore, I postulate that the decrease of IFN-γ producing splenocytes seen with the higher doses was due to a high localized concentration of β_2m.

Our vaccine construct was being driven by the enhancer-promoter sequences from the immediate early gene of the human cytomegalovirus (CMV), thus higher expression levels of β_2m would be expected than under normal conditions. Furthermore, β_2m

naturally presented itself on the outside of the cell in a non-covalent manner bound to the MHC heavy chain in an interaction known to be tenuous (Karlsson, Groth et al. 1980). This combination makes it relatively easy to imagine the existence of high localized concentration in the area where the Sendai epitope was being presented. This high localized concentrations of β_2m would have also been seen in the 'matrix design' experiment, especially with groups 4, 6, and 9 where more than 300 μg of a β_2m-expressing vaccine was injected.

This theory that high localized concentrations of β_2m were a problem could easily be tested. An experiment similar to the one proposed to determine if high doses of DNA were an issue could be performed. However, instead of supplementing the doses of β_2mL21SESP with the pVAX 1 vector, one could simply use the β_2mL21DMSP vector, since this vector also produces β_2m. This experiment could provide evidence towards making the case that high-localized concentrations of β_2m were a legitimate concern. Of course the experiment with the pVAX 1 vector would have to be performed as a control to rule out the potential effect of the high doses of DNA.

If high localized concentrations of β_2m were established as a problem with this vaccine, then changing the route of delivery from IM to intranasal (IN) would be advisable. IM administration provided a convenient initial route to test the vaccines; it was fairly easy to perform without having to anesthetize the mice and has been proven to elicit systemic immune responses. Although mice would need to be anesthetized, the IN route has been shown to elicit both systemic and generalized mucosal immune responses (Oh, Kim et al. 2001; Tadokoro, Koizumi et al. 2001; Iqbal, Lin et al. 2003). Following DNA vaccination via the IN route Oh et al, 2001, showed mRNA corresponding to the DNA vaccine could be found in the lung, spleen, brain, and lymph nodes (Oh, Kim et al.

2001). The widespread distribution of the vaccine might overcome high localized β_2m concentrations.

STUDIES WITH LIVE SENDAI VIRUS

Given that a 400 μg dose of DNA was used for both the priming and boosting in the protection studies, high localized concentrations of β_2m possibly existed in these animals. While β_2m concentrations in the hind leg muscle and associated draining lymph node might prevent priming of the immune system, it should have little effect on the generation of an immune response in the lungs. Therefore, it might be hard to argue that the concentration of β_2m had a negative effect in the protection study. Furthermore, if the vaccines were unable to prime the CTL response ahead of the infection then these mice should have responded similarly to the unprimed mice. Another concern for this vaccine might be that the β_2mL21SESP vaccine inappropriately activated the clonal expansion of a viral specific CTL population that recognized the Sendai epitope but was unable to respond effectively. In this situation, the mice in this group would have faired poorly while the mice in the dummy vaccinated group would have responded in the same manner as the unvaccinated group. However, in this study all vaccinated mice, including the dummy vaccinated ones, faired worse than the unvaccinated control mice. As a group they showed an increased weight loss and decreased survival numbers.

Aside from the injection of a plasmid expressing β_2m, all vaccinated mice received 25 μg of a plasmid expressing the IL-12 cytokine. IL-12 was included because it is known to shift the immune response to favor the secretion of T_H1 cytokines, such as IFN-γ (Kim, Ayyavoo et al. 1997; Scott and Trinchieri 1997; Park and Scott 2001; Trinchieri 2003). Since CTLs respond better in a T_H1 cytokine environment, directing

the immune system in this direction was believed to be a desirable end result. Clear evidence of the ability of IL-12 to increase the number of splenocytes secreting IFN-γ can be seen by the difference observed when mice were given β_2mL21SESP plasmid alone as compared to those that also received the IL-12 plasmid (Figure 6.29). However, unless the high β_2m concentrations were not just a localized event but instead had widespread consequences then the only other possibility consistent with the data available was that the IL-12 was having a negative effect.

The co-administration of IL-12 as an adjuvant with a DNA vaccine has shown mixed results that depend on the virus the vaccine is trying to protect against. Co-administration of IL-12 has been shown to increase the protective characteristics of vaccines generated against pseudorabies virus, Mycobacterium tuberculosis, herpes simplex virus-2, hepatitis B, and the parasites *Leishmania major* and *Trypanosoma cruzi* (Kim, Ayyavoo et al. 1997; Chow, Chiang et al. 1998; Mendez, Gurunathan et al. 2001; Katae, Miyahira et al. 2002; Triccas, Sun et al. 2002; van Rooij, Glansbeek et al. 2002). The use of IL-12 as an adjuvant had no effect on DNA vaccines against influenza (Iwasaki, Stiernholm et al. 1997; Lee, Youn et al. 1999). Finally, IL-12 as an adjuvant has been shown to have negative effects on vaccines designed to protect against Japanese encephalitis virus JAV, SIV when a cDNA library was used as the vaccine, and Feline infectious peritonitis (FIP) (Chen, Pan et al. 2001; Glansbeek, Haagmans et al. 2002; Sykes, Lewis et al. 2002). While, JAV generally requires the production of neutralizing Ab for protection, which would not be favored by a shift towards an T_H1 response, SIV and FIP rely on a cellular immune response to help provide protection. Also, Romani, Puccetti, and Bistoni, in a review, have stated that "in acute viral or fungal infections, the immuno-toxicity of relatively low doses of IL-12 was associated with the uncontrolled production of pro-inflammatory cytokines, such as TNFα and IFN-γ, thus resulting in

septic shock-like pathology or excessive sensitization to microbial stimuli" (Romani, Puccetti et al. 1997).

As far as I know the present work has been the first study using IL-12 as an adjuvant with a DNA vaccine against the Sendai virus. Therefore, there exists a possibility that IL-12 might have a negative effect on the protection against the Sendai virus. Unfortunately, prior to performing the protection study, it was not anticipated that IL-12 or high concentrations of β_2m might present a problem, thus no independent controls were incorporated into our experiment. For this reason the negative effect seen in the vaccinated group cannot be attributed to one or the other possibilities without performing additional experiments.

When comparing the groups in the protection study that were given IL-12, the group that faired best was the mice vaccinated IM with β_2mL21SESP and boosted SC with pβ_2mL21SE. The vaccination strategy of using a DNA vaccine to prime the system and a protein vaccine to boost has shown promise by others (Letvin, Montefiori et al. 1997; Habel, Chanel et al. 2000; Hammond, Jansen et al. 2001; Hevey, Negley et al. 2001). Letvin et al., 1997, showed that rhesus monkeys vaccinated with a vector encoding for the HIV Env protein and boosted with the recombinant Env protein were protected from a chimeric SIV challenge (Letvin, Montefiori et al. 1997). It would, however, be premature to claim that this vaccination strategy was the reason for decreased weight loss seen in mice from this group in the protection study or for that matter the increased number of IFN-γ producing splenocytes seen in mice boosted with the peptide vaccine (Figure 6.30). The reason it would be premature is that the effect of only having an initial priming with the DNA vaccine and no boost whether it be protein or DNA has not been investigated. The combination of high localized concentrations of β_2m plus a subcutaneous injection of the recombinant β_2m-Sendai epitope fusion protein,

which should not increase the localized concentration of β_2m in the muscle tissue or popliteal node, provides a way for the peptide boost to show a false positive effect.

The protection study will need to be repeated with and without IL-12. Before conducting the experiment it would be prudent to investigate administering the vaccine intranasally (IN). Also, it would be advisable to determine the cause for the decreased response observed with higher doses of DNA and then make the necessary adjustments to the protection study to account for it. Additionally it will be necessary to determine a dose of Sendai virus that is lethal to unprotected mice.

In the tetramer staining and viral titer studies, performed with sub-lethal doses of Sendai virus, the only controls were mice vaccinated with the dummy epitope and with the IL-12 plasmid. Therefore no information concerning the negative effects of the IL-12 plasmid or high β_2m can be gained from these experiments. However, in these experiments, along with the comparative results in the protection study, the β_2mL21SESP vaccinated mice mounted a better viral specific immune response than did the dummy vaccinated group. The β_2mL21SESP vaccinated group mounted a viral specific immune response in the lungs by day 4, whereas this response was not seen in dummy-vaccinated mice until day 7 (Figure 6.34). Throughout the study the β_2mL21SESP group showed higher percentages of $CD8^+$ viral specific memory cells in the lungs than did the controls. Increased percentages of $CD8^+$ viral specific memory cells were also observed in the spleen, MLN, and BAL of β2mL21SESP vaccinated mice compared to the dummy vaccinated group (Figures 6.35-6.37). Additionally, based on viral titers, by day 10 all three mice in the β_2mL21SESP vaccinated group had cleared the infection from the lungs while only one of the three mice in the β_2mL21DMSP group tested negative for virus (Figure 6.38).

CONCLUSION

Unfortunately questions still exist as to the protective ability of this vaccine, therefore it would be premature to suggest that the vaccine could one day be used as either a prophylactic or therapeutic treatment for HIV. Assuming the problems with the protection study were due to the co-administration of IL-12, modification would still be required to make it effective against HIV. First, when designing an HIV version of this vaccine the murine β_2m will need to be exchanged for the human version of this molecule. Second, an appropriate HIV epitope will need to be identified. HIV epitopes should be chosen based on their ability to be presented in different MHC-I alleles along with their likelihood of being broadly cross-reactive against the different stains of HIV. Many epitopes have been identified that show these characteristics: for example the Gag – p17, residues 77-85, Pol, residues 313-321, and Nef, residues 71-80 (Ferrari, Kostyu et al. 2000). The basic design allowing for the easy exchange of epitopes should be kept to make it straightforward to design multiple vaccines covering a spectrum of HIV CTL epitopes.

Overall this work has made a novel contribution to the understanding of β_2m viral epitope fusion vaccines. The choice of linker size has been shown to be an important consideration when tethering the β_2m to the viral epitope. The ability of a DNA vaccine to generate a viral specific immune response has been established. Though the protective ability of the vaccine has not been established, the early arrival of viral specific memory cells gives credence to the vaccine's potential to provide protection.

Thank You For Your Support, Good Bye and Good Luck.

APPENDIX

Appendix I: Materials and Methods

PROTEIN VACCINE PROTOCOLS

Extension PCR

This protocol uses the Expand High Fidelity PCR System (Roche) to create the PCR products that code for the β_2m-Sendai epitope fusion proteins. For the pβ_2mL10SEHT and pβ_2mL15SEHT constructs, the first template was the pSK-β_2m plasmid and the second was the purified PCR product from the reaction with the first template. For the pβ_2mL15DMHT the only template used was the pβ_2mL15SEHT pCRT7/NT plasmid. The primers can be found in the section Appendix: Primers.

___1. Prepare the PCR cocktail below

MM1		µl	MM2		µl
dNTPs (Roche)	10mm	2	Expand buffer 2	10X	10
T7 primer	15µm	2	dH_2O		39
Upstream Primer	15µm	2			
Template		2			
dH_2O		42			

___2. Make 2 sample mixtures of MM1 and MM2 by mixing 25µl of MM1 with 24.5 µl of MM2 in a small PCR tube then add 0.5 µl of Expand PCR enzyme (taq + pfu). Samples now have a total volume of 50 µl.

___3. Run PCR program danny β2m on the Perkin Elmer GeneAmp PCR System 2400 (Figure A.1)

___4. Run a 1% agarose TAE gel at ~75 V for 1 hr on PCR product to verify amplification.

___5. Purify the remaining PCR product using the PCR purification kit (Qiagen)

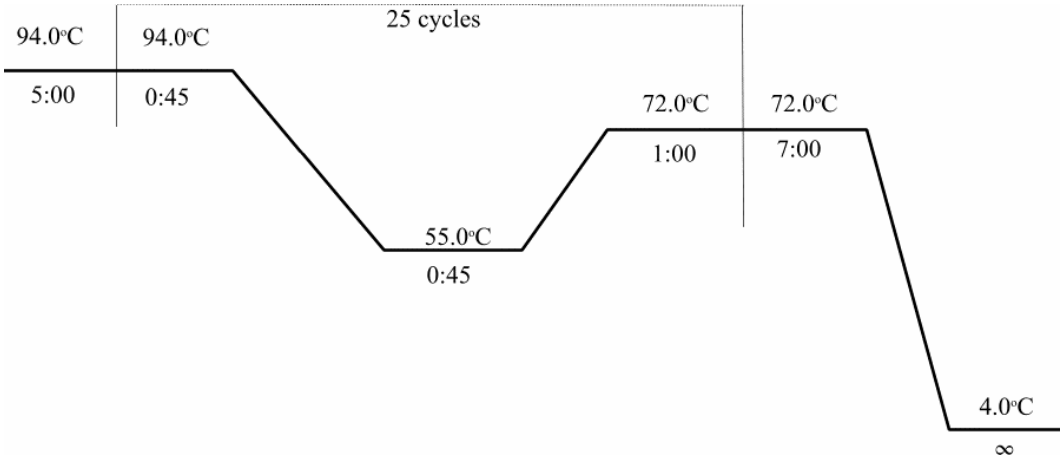


Figure A.1 PCR program danny β2m

TOPO cloning for the pβ$_2$mL10SEHT, pβ$_2$mL15SEHT and pβ$_2$mL15DMHT

This protocol was use to transform a clone bacteria with the vectors expressing pβ$_2$mL10SEHT, pβ$_2$mL15SEHT and pβ$_2$mL15DMHT, respectively.

___1. The purified PCR product was then ligated into the pCRT7/NT-TOPO vector following the directions provided in the pCRT7 TOPO TA Cloning kit version E (Invitrogen) page 11 (Invitogen 2000). (See below)

___2. The cells were allowed to recover in 200 µl of SOC (below) in the 37°C New Brunswick Scientific C25 Incubator Shaker at 37°C for 30-45 minutes at 230 RPM. Avoid incubating the cells in SOC for longer than 60 minutes; since the SOC media does not contain a selection antibiotic longer incubation times may cause the bacteria to reject the plasmid.

___3. While waiting on the cells to recover, LB plates containing 60 µg/ml ampicillin should be placed into the 37°C oven to warm.

___4. Using two different amounts (one small ~ 50 µl, one large ~ 180 µl), plate the SOC/cell sample on to the two LB plates. Spread the samples evenly and then let them set face up for 10 minutes in the 37°oven.

___5. After 10 minutes flip the plates and let incubate overnight.

For SOC media: dissolve 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 950 ml of dH_2O, add 10 ml of 250 mM KCl and pH to 7.0 and fill to 1 L with dH_2O, autoclave then add 20 ml of a 1 M glucose solution that had been passed through a 0.22 micron filter. Aliquot into 200 µl samples and store at -80°C)

pCRT7 TOPO TA Cloning kit version E (Invitrogen) page 11 (Invitogen 2000)

This protocol was used to ligate the PCR products into the pCRT7/NT TOPO vector.

___1. Turn on water bath to 42ºC and thaw of SOC (above) to room temperature.

___2. Prepare the reaction below

Fresh PCR product	0.5-4µl
Salt Solution	1µl
Sterile Water	add to final volume of 5µl
TOPO Vector	1µl
Total	6µl

___3. Mix reaction gently and incubate for 5 minutes at room temperature, then place mixture on ice.

___4. Add 2µl of TOPO reaction from above into a vial of One Shot Chemically Competent *E. coli* and mix gently. DO NOT mix by pipetting up and down.

___5. Incubate on ice for 5-30 minutes.

___6. Heat-shock the cells for 30 seconds in a 42ºC water bath without shaking.

___7. Immediately transfer the tubes to ice.

Master plate for all constructs

This protocol was used to create a plate of isolated colonies.

___1. Draw a 4x4 grid on the back of a clean LB plate containing 60 μg/ml ampicillin.

___2. Using a platinum wire tool, pick a colony off the original plate and place it down in one of the squares created on the fresh plate. Repeat this process until all squares are occupied. 16 colonies were transplanted.

___3. Then place the new plate face up for 10 minutes in the 37°oven, after 10 minutes flip the plates and let incubate overnight

Colony PCR screening for all constructs

This protocol was used to determine if transformed bacteria cells had a plasmid with the desired length for the inserted construct. The primers can be found in Appendix: Primers.

___1. Prepare the PCR cocktail below to screen 10 colonies.

		μl
dNTPs	10mm	6
Forward primer	15μm	6
Reverse primer	15μm	6
Taq Buffer (Roche)	10X	22
dH_2O		180
total		220

___2. Mix well then, divide the cocktail into 10 labeled tubes, 20 μl per tube.

___3. Take a swipe of a colony with a toothpick, and swirl it in the PCR cocktail.

___4. Boil the cocktail for 5 minutes.

___5. Transfer the cocktail to PCR tubes.

___6. Add 0.5 µl of Taq polymerase (Roche)

___7. Run the Danny β2m program on the Perkin Elmer GeneAmp PCR System 2400

___8. Add 4 µl of 6X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in dH_2O) to each tube and run a 1% agarose TAE gel at 75V for ~1.5 hrs to see if the amplified band is present. Running buffer is 40 mM Tris-acetate, pH 8.0, 1 mM EDTA.

Protein expression and purification

This protocol was used to obtain large amounts of pure pβ2mL21SE, $p\beta_2$mL15SE, $p\beta_2$mL10SE, or $p\beta_2$mL15DM.

Heat shock transformation

___1. Turn on water bath to 42°C

___2. Combine 1 µl of pure plasmid with 20 µl of BL21(DE3) cells

___3. For a negative control combine 1 µl of dH_20 with 20 µl of cells

___4. Store cells on ice for 20-30 min

___5. Thaw out one 200 µl vial of SOC for every transformation

___6. Incubate cells in 42°C bath for 30-60 sec.

___7. Put cells back on ice for 2 minutes

___8. Add 200 µl of SOC to each vial of cells

___9. Place vials in the incubator shaker at 37°C for 45-60 minutes at 230 RPM.

Growth, Induction, and Harvesting

___10. With the SOC/cell solution inoculate 10 ml of LB with 60 μg/ml ampicillin, do the same for the negative control. To ensure proper oxygen delivery, choose a tube where the total volume of liquid is no more than 30% of the total volume of the container.

___11. Place in the 37°C incubator shaker overnight at 230 RPM

___12. With 3 ml of the overnight culture of cells inoculate two 6L flask containing 1400 ml of LB with 60 μg/ml ampicillin.

___13. Let cells grow to an OD_{600}= 0.5-0.8 in the incubator shaker at 37°C and 230 RPM.

___14. Induce the expression of the insert with 1 mM final concentration Isopropylthio-β-D-galactoside (IPTG)

___15. Allow protein expression for 2-3 hours in the incubator shaker at 37°C and 230 RPM.

___16. Harvest cells at 4500g for 15-20 min at 4°C with Sorvall GSA rotor in the Sorvall RC-5B Refrigerated Superspeed Centrifuge.

___17. Collect and weigh cell pellet

___18. Store cell pellet at -80°C until needed.

Cell Lysis and Ni NTA Column

___19. Thaw out cell pellet

___20. Lyse cells in 5 times volume/pellet weight (ml/g) with Lysis Buffer B (8M urea, 100 mM NaH_2PO_4, 10 mM Tris•Cl (pH=8.0)).

___21. Mix solution on a stir plate for 1-1.5 hrs

___22. Equilibrate a charged Ni-NTA agarose column (QIAGEN) with Lysis Buffer B, the column size should be 1 ml column per 20 g of cell pellet.

___23. To remove cell debris spin the lysed cell solution at 27000g, 4°C for 20 min in the Sorvall SS-34 rotor in the Sorvall RC-5B Refrigerated Superspeed Centrifuge.

___24. Load supernatant onto the Ni-NTA column and discard pellet

___25. Washed with Lysis Buffer B, pH=6.3, equal to the amount loaded.

___26. Eluted with the Lysis Buffer B, pH=4.5 equal to ~15% of the load volume

___27. Adjust pH of eluate to ~ 8 to reduce problems associated with lower pHs.

___28. Run a 15% Tris-Glycine SDS-PAGE gel at 200 V for ~ 40 min to check the purity of the eluted protein

Refolding

The protein was refolded in a two-step dialysis process similar to that described in the Current Protocols in Protein Science page 6.5.3-4 (Coligan, et al., 2002).

___29. Tie a double knot into one end of wet 6,000-8,000 dialysis tubing (Spectrum)

___30. Transfer the eluted protein into the dialysis tubing and double knot the other end

___31. On a stir plate dialysize against 4 liters BHG buffer A (50 mM glycine, 10% (w/v) sucrose, 1 mM EDTA, 4 M Urea, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, pH 9.6) overnight at 4°C.

___32. Remove the tubing from BHG buffer A and dialyze against BGH buffer B (60 mM ethanolamine, pH 9.6, 10% (w/v) sucrose, 1 mM EDTA, 0.1 mM reduced glutathione, 0.01 mM oxidized glutathione) for 6-8 hrs on the stir plate at 4°C

___33. Perform BCA Protein Assay (Pierce) according to the manufacturer's instructions to determine the concentration of the protein

Poly-histidine tag removal and protein concentration.

___34. Incubate up to 10 mg of protein, as determined from a BCA Protein Assay, with 50 U recombinant Enterokinase (Novagen) for 16 hours in BGH buffer B.

___35. Concentrate the cut protein using a YM-3 Centriprep (Amicon) spun at 3000g, 4°C in a GSA rotor.

___36. To exchange the BGH buffer B with PBS; when the protein sample volume in the previous step reaches 1 ml add 14 ml of PBS and concentrate again, repeat. This is equivalent to dialyzing 30 ml against 6.75 liters.

___37. Perform BCA Protein Assay according to the manufactures instructions to determine the concentration of the protein

Upregulation of MHC I expression

The upregulation experiment was used to determine if the β_2m-Sendai epitope fusion proteins could bind to the MHC heavy chain and stabilize the MHC-I complex on the outside of RMA-S cells.

___1. Grow RMA-S cells in RMA-S Growth Media ((RPMI 1640 with L-Glutamine containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) termed RGM) at 37ºC in a 5% CO_2 humidified incubator.

___2. Take twice as much protein as the total to be used in the incubation with RMA-S cell. For example, for five 100 μl 50μM samples, use the equivalent of 10 samples. (later half the sample will be lost) Exchange the PBS that the proteins are in with RGM, using a Centricon 3 or Microcon 3 by combining 20x the volume of protein with the media. Spin time is ~3 hours at 1315g and 4°C in the Adams Compact II Centrifuge (Becton Dickinson) (Dr. Kramers cold room).

Concentrate the samples to below twice the total volume need for incubation. For example that would be below 1000 µl. (Note it is likely that the antibiotics got filtered out, so be sure to use sterile techniques)

Perform steps 3-7 and 9-11 under the laminar flow hood with sterile techniques

___3. Bring the sample volume to twice that used need for incubation with media. For our example that would be 1000 µl. So you should have 1000 µl of a 50 µM sample.

___4. Pass the samples through a .22 µm syringe filter, collect all the sample you can. (this is where you will lose about half the sample)

___5. Combine 990 µl of RGM with 10 µl of 5mM peptide (FAPGNYPAL, synthesized from the University of Texas Protein Facility) to make a 50 µM solution and pass this through a .22 µm syringe filter.

___6. Count the RMA-S cells using a hemocytometer

___7. Take $3x10^5$ cells per sample per protein and combine the sample for the same protein together in a 2.0 ml microfuge tube.

___8. Spin the cells at 510g for 5 min in the Eppendorf Centrifuge 5412c in the 4°C cold room

___9. Carefully remove the supernatant using a pipette

___10. Add the RGM containing the protein or peptide control to the cells and mix by pipetting up and down a couple of times.

___11. Put 100 µl of cells per well into a 96 well plate.

___12. Place the plate into a 30°C incubator with 5% CO_2 for one hour

___13. Seal in the CO_2 using Parafilm and place the plate at 26°C for 18-22 hrs

___14. Remove the Parafilm and place the plate in a 37°C incubator with 5% CO_2 for 2 hr

From this point on keep everything on ice.

___15. Remove the plate and transfer the cells to labeled 0.6 ml microfuge tubes

___16. Pellet the cells at 510g for 5 minutes in the Eppendorf Centrifuge 5412c in the 4°C cold room

___17. Remove the supernatant using a pipette.

___18. Add 200 µl of PBS with 0.1 % sodium azide and flick the tube several times to resuspend the cell pellet

___19. Pellet the cells at 510g for 5 minutes at 4°C.

___20. Remove the supernatant using a pipette.

___21. Add 200 µl of PBS with 0.1 % Sodium Azide and 2.0 µg/ml of R-PE Anti-mouse H-2K^b Ab and then flick the tube several times to resuspend the cell pellet

___22. Let the sample incubate on ice for 30 min

___23. Pellet cells at 510g for 5 minutes at 4°C.

___24. *** Wash twice with 200 µl of PBS with 0.1% Sodium Azide by pelleting and removing the supernatant.

___25. After both washes resuspend the pellet in 200 µl of PBS with 0.1% Sodium Azide and store on ice until Flow cytometry, (can be stored without fixing for no more than a few hours)

___26. Run the flow cytometer (BD FACSCalibur) in a log scale for collecting the PE fluorescence, because it will max out the linear scale. Report the mean fluorescence data. (note dead cells will have higher side scatters and lower forward scatters, thus try to gate them out)

*** Using a dye such as 7-Aminoactinomycin D (7AAD) or To-Pro3 to stain for dead cells is recommended and should be done after step 23, but this was only done in one of

the upregulation experiments. Simply, remove the cells from the ice, add 2 µl of 7AAD solution (BD Pharmingen) per 100 µl of sample, incubate at room temperature in the dark for 10 min, run FACS.

Tumor cocktail and complete tumor media (CTM) preparation

This media was used with the T-cell hybridoma clones.

___1. In a large sterile flask mix

MEM	Gibco 320-1380AJ	630 ml
Dextrose	Gibco 15023-021	7.5 g
Essential Amino Acids (50x)	Gibco 11130-051	75ml
Non-Essential Amino Acids (100x)	Gibco 12383-014	140 ml
Sodium Pyruvate (100x)	Gibco 11360-070	100ml
Penicillin/Streptomycin/Glutamine (100x)	Gibco 10378-016	100 ml

___2. Adjust pH to 7.0 with 10 N NaOH

___3. Then add:

Sodium bicarbonate	Gibco 11810-025	8.5 g
β-mercaptoethanol	Gibco 21985-023	34 µl

___4. Filter the solution through a 500 ml .22µm filter PES vacuum flask

___5. Aliquot 43ml into sterile 50 ml tubes and freeze.

___6. Add 43 ml to 500 ml of S-MEM + 10% FBS to make tumor media

In the original protocol from David Woodland's lab 500 mg of Gentamycin sulfate was used, however I have omitted this antibiotic because we did not have it. Also I felt like it was overkill because there are already two antibiotics, Penicillin and Streptomycin, in the mix.

T-cell hybridoma stimulation assay for peptide vaccines

This protocol was intended to determine if any of the linkers interfered with T-cell receptor recognition. However, the T-cell hybridoma stimulation assay proved to be inappropriate for this task, thus eventually all peptide vaccines were tested *in vivo*.

___1. Grow RMA-S cells in RGM in a 5% CO_2 humidified incubator.

___2. Exchange the PBS that the proteins are in with RGM, using a centricon 3 or microcon 3. Described above in "Upregulation of MHC I Expression". Final concentration of the vaccine should be 50 μM

___3. Prepare a 50 μM solution of Sendai peptide as described in "Upregulation of MHC I Expression"

___4. Count the RMA-S cells and put 4.5 samples of $3x10^5$ cells per well into a 15ml falcon tube and spin down at 400g in the ThermoIEC Centra CL2 clinical centrifuge for 5 min.

___5. Resuspend in the appropriate media containing the vaccine or peptide

___6. Plate 4 wells of $3x10^5$ cells per well in a 96 well plate

___7. Place the plate into a 30°C incubator with 5% CO_2 for one hour

___8. Seal the CO_2 in using Parafilm and place the plate a 26°C for 18-20 hours

___9. After the incubation, pellet the cells at 4°C for 5 minutes at 400g in the Beckman CS-15R centrifuge with the S2096 Rotor.

___10. Carefully remove supernatant.

___11. Added 200 μl of the T-cell hybridomas in CTM at a total of $4.5x10^5$ hybridoma cells per well. This can be individual hybridoma clones or a combination of clones.

___12. Pipette cells up and down several times

___13. Incubate at 37°C and 5% CO_2 for 18-24 hours.

___14. Perform IL-2 ELISA.

IL-2 ELISA

This protocol follows the procedure outlined by the manufacturer for the OptEIA Mouse IL-2 Set (BD Pharmingen). The recombinant IL-2 and antibodies used were from this set. This experiment was used to determine the amount of IL-2 released during T-cell hybridoma stimulation assays.

___1. Coat microwells with 100 µl per well of Capture Antibody (Anti-mouse IL-2 monoclonal antibody) diluted 1:500 in coating buffer (0.1M Carbonate, pH 9.5 made by dissolving 0.84g $NaHCO_3$ and 0.356 g Na_2CO_3 adjusting the pH to 9.5 and filling to 100 ml).

___2. Seal plate and incubate overnight at 4°C.

___3. Discard coating solution and wash 3 times with ~300 µl of wash buffer (PBS, pH 7, with 0.05% Tween-20)

___4. Block Plates with ~200 µl of assay diluent (PBS, pH 7, with 10% FBS) for 1 hour at room temp.

___5. While the plate is blocking transfer samples to 0.6 ml microfuge tubes

___6. Pellet cells at 510g for 5 minutes in the Eppendorf Centrifuge 5412c in the 4°C cold room

___7. The supernatant will be assayed for IL-2.

___8. Prepare IL-2 200 pg/ml standard by following instructions on Instruction/Analysis Certificate, which were; add 10 µl of recombinant mouse IL-2 to 6.44 ml of assay diluent and mix.

___9. Prepare 300µl of the other standards (100, 50, 25, 12.5, 6.3, 3.1 pg/ml) by serial dilutions of the 200 pg/ml sample supplementing with assay diluent. Use assay diluent as the zero standard.

___10. Discard blocking solution and wash wells 3 times with ~300 µl wash buffer.

___11. Pipette 100 µl of each sample supernatant or standard into the appropriate wells.

___12. Seal plate and incubate for 2 hrs at room temperature.

___13. Discard sample and standard solutions and wash wells 5 times with ~300 µl wash buffer.

___14. Add 100 µl of working detector (1:2000 dilution of detection antibody, biotinylated anti-mouse IL-2 in assay diluent plus avidin-horseradish peroxidase conjugate at a 1:500 dilution)

___15. Seal plate and incubate for 1 hr at room temperature.

___16. Discard the working detector solution and wash wells 7 times with ~300 µl wash buffer. Allow the wash to soak for at least 30 seconds during each wash.

___17. Add 100 µl of 1-Step Ultra TMB substrate solution (Pierce)

___18. Incubate plate in the dark until desired color is achieved, usually 30 min to 1 hour.

___19. Add 50 µl of stop solution (0.5M oxalic acid)

___20. Read absorbance with the EL340 Microplate Bio Kinetics Reader (Bio-Tek Instruments) at A 450 nm within 30 minutes of stopping reactions. (You may chose to perform a wavelength correction by subtracting the A 570 nm from the A 450 nm)

ELISPOT assay

This protocol follows the procedure outlined in the manufacturer's instructions for the Mouse IFN-γ ELISPOT Set (BD Pharmingen) and was used for all ELISPOT to determine the number of INF-γ secreting cells.

Under the Laminar Flow Hood

___1. Dilute capture antibody, purified anti-mouse IFN-γ (BD Pharmingen), 1:200 in coating buffer (PBS, pH 7.2, sterile filtered)

___2. Add 100 µl of dilute antibody solution to each well of an ImmunoSpot Elispot plate (BD Pharmingen). (These plates contain a special membrane necessary to perform the assay. Take care not to puncture the membrane when adding reagents to the plate.)

___3. Seal plate and store at 4°C overnight in the tissue culture refrigerator

___4. Aspirate off coating buffer and wash wells once with blocking solution (RPMI 1640 with L-Glutamine containing 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin (i.e. RGM)

___5. Add 200 µl of blocking solution to each well and incubate at room temperature for 2 hr.

___6. During the blocking time, count the cells you want to add.

___7. Make the necessary dilutions to give a final cell count of $2x10^6$ cells/ml.

___8. Perform serial dilutions to give cells concentrations of $2x10^6$ cells/ml, $1x10^6$ cells/ml, $5x10^5$ cells/ml and $2.5x10^5$ cells/ml.

___9. Aspirate off blocking solution

___10. Added 100 µl of 100 µM Sendai Peptide in growth media to each well, final concentration 50 µM (except Sendai peptide minus negative controls)

___11. Added 100 µl of cells to each well following the 96 well format below.

___12. Replace lid and incubate plates at 37°C in 5% CO_2 for between 18-24 hr.

Sterile conditions no longer required

___13. Discard cells and wash wells 2x with deionized water. Allowing each wash to soak for 3-5 minutes.

___14. Wash wells 3x with wash buffer I (PBS, pH 7.2, with 0.05% Tween-20) letting buffer soak for 2 min

___15. Dilute detection antibody, biotinylated anti-mouse IFN-γ (BD Pharmingen), 1:250 in dilution buffer (PBS, pH 7.2, with 10% FBS)

___16. Add 100 µl to each well.

___17. Replace lid and incubate at room temperature of 2 hrs.

___18. Discard detection antibody solution and wash wells 3x with wash buffer I letting buffer soak for 2 min

___19. Dilute avidin-horseradish peroxidase 1:100 in dilution buffer.

___20. Add 100 µl to each well.

___21. Replace lid and incubate at room temperature for 1 hr.

___22. Discard avidin-HRP solution and wash wells 4x with wash buffer I letting buffer soak for 2 min.

___23. Wash well 2x with PBS letting buffer soak for 2 min

___24. Add 100 µl of final substrate solution (see below)

___25. Monitor spot development from 5~60 minutes. Usually closer to 60 minutes.

___26. Stop substrate reaction by washing wells with DI water.

___27. Air dry plates for at least 2hrs (overnight okay) in the dark.

___28. Store plates in the dark until analysis.

___29. Count spots under a Olympus SZX12 microscope (located in Dr. Tesmer's cold room).

Final substrate solution preparation.

___30. Prepare AEC (3-amino-9-ethyl-carbazole; Sigma A-5754) stock solution by dissolving 100 mg of AEC in 10 ml of DMF (N,N-dimethylformanide; Sigma D-4551) ** Prepare solution in glassware under the fume hood because it will react with plastics.

___31. Prepare 0.1 M acetate solution by combining 14.8 ml of 0.2 M acetic acid/glacial acid with 35.2 ml of 0.2 M sodium acetate, adjust pH to 5.0 and fill to 100 ml.

___32. Now combine 333.3 µl of AEC stock solution with 10 ml of 0.1 M acetate solution and filter through a 0.45 µm filter. Add 5 µl of H_2O_2 (30%) and use immediately.

DNA VACCINE PROTOCOLS

Extension PCR for DNA constructs

See PROTEIN VACCINE PROTOCOLS. This experiment was used to create the DNA constructs β_2mL10SESP and β_2mL15SESP. For both the first template was the pSK-β_2m vector; the second template was a purified first PCR product with added the linker; the third template was a purified second PCR product which added the Sendai epitope and a portion of the Signal peptide. The primers for these reactions can be found in the section, Appendix: Primers.

Restriction digestion of DNA construct (PCR product) and pVAX1 plasmid

This protocol was used to create the appropriate overhangs in the pVAX1 plasmid and final DNA PCR products for the β_2mL10SESP and β_2mL15SESP. The overhangs will facilitate in the ligation process.

Ethanol precipitation

If the DNA is in a solution other than water then ethanol precipitate the DNA prior to digestion.

___1. Transfer DNA to a container where it fills no more than one fourth the total volume. For example, a 2000 µL tube should have no more than 500 µL of DNA solution.

___2. Add one tenth volume of sodium acetate buffer to equalize ion concentrations

___3. Add at least two volumes of cold 100% ethanol; let stand in -20°C freezer for at least one hour (okay to go overnight)

___4. Centrifuge sample for 15 minutes at >18,000g on the Eppendorf Centrifuge 5412c in the 4°C cold room

___5. Remove as much supernatant as possible with a 1 ml micropipet; recentrifuge for 1 min, then remove the rest with a 200 µL pipet

___6. Add 200 µL of cold 70% ethanol; centrifuge for 5 minutes in the centrifuge at 4 °C

___7. Remove supernatant with a 200 µL pipet; evaporate remaining ethanol in a 37 °C water bath or at room temperature.

___8. Resuspend pellet in desired volume of water.

Sequential digestion

___9. Turn on the water bath and set it at 37°C.

___10. Pipette the following amounts into vials, all values are µl.

	pVAX1	PCR3	Xho I*	Nhe I	No Cut
Buffer M	4	4	1	1	1
pVAX1 Vector (0.33 µg/µl)	3	0	1	1	1
PCR product	0	33	0	0	0
Sterile dH_2O	30	0	7	7	8
Xho I ** (New England Biolabs)	0	0	1	0	0
Nhe I ** (New England Biolabs)	3	3	0	1	0
Total	40	40	10	10	10

* Prepare Xho I at step 13

** Add the enzymes last, right before your are ready to incubate the reaction. Always try to minimize the time the enzymes are out of the -20°C freezer.

___11. Let digest 1.5 hrs.

___12. Inactivate the enzymes by heating to 65ºC for 20 min

___13. Prepare the Xho I control, after the Nhe I digestion.

___14. Add 2 μmoles Tris-HCl (pH 7.5) to the pVAX1 and PCR sample to bring the final concentration (fc) to about 50 mM. So add 2 μl of 1 M Tris-HCl pH 7.5.

___15. Add 2.6 μmoles NaCl to the pVAX1 and PCR sample to bring the fc ~100 mM. So add 2.6 μl of 1 M NaCl.

___16. Add 3 μl of Xho I to the pVAX1 and PCR.

___17. Incubate the reactions for 1.5 hrs in the water bath at 37°C.

___18. Run the entire pVAX1 and PCR product on a 1% agarose TAE gel with the following lanes assignments, instead of adding 6X loading dye to the PCR use 30% glycerol as a 6X loading buffer.

___19. Cut the pVAX 1 and PCR product out and purify using the QIAEX II gel extraction kit. Eluded with dH_2O for sequencing or 10 mM Tris·Cl, pH 8.5 for storage.

___20. Run a 1% agarose TAE gel to determine the concentration of each.

Restriction digestion of pVAX1 β_2mL15SESP to cut out Sendai epitope.

This protocol was followed to cut out the Sendai epitope form pVAX1 β_2mL15SESP so that the β2mL21SESP, β2mL21DMSP, and β2mL21NESP plasmids could be created. Start by Ethanol Precipitate the pVAX1 β_2mL15SESP, if needed. See Restriction digestion of DNA construct (PCR product) and pVAX1 plasmid.

Sequential restriction digestion

___1. Turn on the water bath and set it at 37°C.

___2. Pipette the following amounts into vials, all values are µl.

	pVAX1	AGE I	BAMH I	No Cut
NEBuffer 1	5	5	5	5
pVAX L15 Vector (0.85µg/µl)	0.59	0.59	0.59	0.59
Sterile dH_2O	40.41	40.41	44.41	44.41
Age I (New England Biolabs)	4	4	0	0
BAMH I (New England Biolabs)	0	0	0	0
Total	50	50	50	50

___3. Mix the reaction by pipetting up and down (do not vortex), then centrifuge to bring solution to the bottom of the tube.

___4. Let digest 1.5 hours at 37ºC.

___5. Inactivate the enzymes by heating to 65ºC for 20 min

___6. Add 2µl of 4M NaCl (final concentration ~ 150mM), 0.53µl of BSA (final concentration = 100 µg/ml), 1µl of BAMH I or 1µl of dH2O

___7. Let digest 1.5 hour at 37ºC.

___8. Inactivate the enzymes by heating to 80ºC for 20 min

___9. Run a 1% agarose TAE gel at 75V for ~1.5 hrs to determine if the DNA was properly cut.

Oligonucleotide annealing

This protocol was used to anneal complementary oligonucleotides that either encode for L21SE, L21DM, or L21NE. The annealed oligonucleotides will be used to create β2mL21SESP, β2mL21DMSP, and β2mL21NESP. The oligos used can be found in the section Appendix: Primers.

___1. Prepare the following mixture in a PCR tube.

	µL	
Oligo 1 10 µM	30	(complementary to oligo 2)
Oligo 2 10 µM	30	(complementary to oligo 1)
Expand 10X PCR buffer (Roche)	10	
dH_2O	30	
Total	100	

___2. In the Perkin Elmer GeneAmp PCR System 2400 heat the sample to 94°C for 5 minutes to separate strands, anneal together at a temperature between 52-56°C for 1 hour

Ligation with Rapid DNA Ligation Kit (Roche)

This protocol was used to ligate the cut final PCR constructs for β_2mL10SESP and β_2mL15SESP into the cut pVAX1 vector. The protocol was also used to ligate the annealed oligonucleotide for the 21 AA linker, the dummy epitope and the no epitope into cut pVAX1 β_2mL15SESP. The concentrations of the insert (cut PCR product or annealed oligos) and the concentration of the cut plasmid are needed to proceed.

___1. In a small vial combine plasmid and insert at a 1 to 3 molar ratio and a 1 to 4 molar ratio plasmid to insert. Make sure the total amount of DNA is less than 200 µg and the total volume less than 8 µl.

___2. For a self ligation control combine plasmid with dH_2O.

___3. Bring volume in above to 8 µl with dH_20.

___4. Add 2 µl of DNA diluting buffer 5x (Vial 2)

___5. Add 10 µl of T4 DNA ligation buffer (Vial 1)

___6. Mix thoroughly

___7. Add 1 µl T4 DNA ligase 5 units/µl (Vial 3)

___8. Mix thoroughly

___9. Incubate at room temp for 20 min

___10. Turn the water bath on to 42°C.

___11. Remove, from the -80°C freezer and thaw on ice, enough DH5-α cells or TOP10F' cells for the reactions, including a negative control.

___12. Remove, from the -80°C freezer and thaw on ice ,1 vial of SOC (200 µl) for each reaction. So for this example that would be 4 reactions: 1/3, 1/4, self ligation control, and negative control.

___13. Add 1.5 µl of the ligation reaction to the chemically-competent 25 µl of bacteria cells and mix gently. For the negative control use 1 µl of dH_2O. Do not pipette up and down. (the negative control verifies that the selection antibiotic works.

___14. Incubate on ice for 20 minutes.

___15. Heat shock the mixture for 30-45 seconds at 42°C.

___16. Immediately put on ice.

___17. Add 200 µl of SOC, and let shake for 1 hr at 37°C.

___18. While you are waiting for the cells to recover, place to LB plates containing 60 µg/ml ampicillin into the 37°C oven to warm them.

___19. Using two different amounts (one small ~ 50 µl, one large ~ 150 µl), plate the SOC/cell sample on to the two LB plates containing 60 µg/ml ampicillin. Spread the samples evenly and then let them set face up for 10 minutes in the 37°oven.

___20. After 10 minutes flip the plates and let incubate overnight.

Colony PCR screening for DNA constructs

See PROTEIN VACCINE PROTOCOLS

DNA purification

This protocol was used to obtain large quantities of β_2mL21SESP, β_2mL15SESP, β_2mL10SESP, β_2mL21DMSP, β_2mL21NESP, and pVAX1.

___1. In the morning, dip a sterile toothpick into the frozen glycerol stock of the bacteria vector with the plasmid of interest and drop it into a starter culture of 10 ml LB with 50 μg/ml kanamycin. (Bacteria glycerol stocks are kept in the -80°C freezer and should never be thawed. Also, always have a primary stock of the isolated plasmid for long term storage). For all bacterial growth samples pick a container where the total volume of solution is less than 30% of the total container volume.

___2. Grow starter culture for 8 hr in the New Brunswick Scientific C25 Incubator Shaker at 37°C and 230 RPM.

___3. Take 3ml of the starter culture and use that to inoculate two 6 L flasks containing 1.2 liters of LB with 50 μg/ml kanamycin. (This is a 1/400 dilution)

___4. Grow bacteria overnight for 12-16 hr in the incubator shaker at 37°C and 230 RPM

___5. Harvest cells at 3000g for 15-20 minutes at 4°C using the Sorvall GSA rotor in the Sorvall RC-5B Refrigerated Superspeed Centrifuge.

___6. Remove the supernatant by inverting the tube and keep the cell pellet. Cell pellets can be frozen at -20°C overnight if need be or at –80 for longer time periods.

___7. Follow directions for steps 4 through 15 in the QIAGEN QIAfilter Handbook for a QIAfilter Giga prep of a high copy plasmid (QIAGEN 2000). A 2000 ml graduated cylinder is convenient container to place the QIAGEN-Tip. For the

pelleting the DNA out of the isopropanol (step 15) the screw top SS-34 tubes work well and can withstand the high g-force.

___8. Add 10 ml of 70% ETOH to the DNA pellet from the isopropanol wash (step 15)

___9. Transfer the ETOH/DNA solution into 2 ml microfuge tubes.

___10. Pellet the DNA at >15,000 g in the Eppendorf Centrifuge 5412c in the 4°C cold room for 10 min.

___11. Remove the supernatant with a pipette

___12. Air dry the pellet for 10-15 min at room temperature.

___13. Add 100-150 µl of PBS to each microfuge tube and resuspend the DNA pellet by votexing

___14. Combine the resuspended DNA into one tube

___15. Determine the DNA concentration using absorbance at 260 nm as measured by the Beckman DU530 Life Science UV/Vis Spectrophotometer. Take 2 µl of DNA and dilute it to 1 ml with PBS then find the A_{260}. DNA concentration equals DNA µg/ml = A_{260} x dilution factor (500 in this example)x50 (µg/ml)/A_{260}. I like reporting as µg/µl so I divided the above value by 1000. Pure DNA solutions will have an A_{260}/A_{280} ratio about 1.8 or higher.

QIAGEN QIAfilter Handbook steps 4-15 (QIAGEN 2000)

Taken from the QIAGEN QIAfilter Handbook

___4. Screw the QIAfilter Mega-Giga Cartridge onto a 45 mm-neck glass bottle and connect it to a vacuum source.

___5. Resuspend the bacterial pellet in 125 ml Buffer P1.

___6. Add 125 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min.

___7. Add 125 ml chilled Buffer P3, and mix immediately and thoroughly by inverting 4–6 times. Mix well until white, fluffy material has formed and the lysate is no longer viscous. Proceed directly to step 8. Do not incubate on ice.

___8. Pour the lysate into the QIAfilter Mega-Giga Cartridge and incubate at room temperature for 10 min.

___9. Switch on the vacuum source. After all liquid has been pulled through, switch off the vacuum source. Leave the QIAfilter Cartridge attached.

___10. Add 50 ml Buffer FWB2 to the QIAfilter Cartridge and gently stir the precipitate using a sterile spatula. Switch on the vacuum source until the liquid has been pulled through completely.

___11. Equilibrate a QIAGEN-tip 2500 or QIAGEN-tip 10000 by applying 75 ml Buffer QBT and allow the column to empty by gravity flow.

___12. Apply the filtered lysate from step 10 onto the QIAGEN-tip and allow it to enter the resin by gravity flow.

___13. Wash the QIAGEN-tip with a total of 600 ml Buffer QC.

___14. Elute DNA with 75 ml Buffer QF.

___15. Precipitate DNA by adding 52.5 ml room-temperature isopropanol (0.7 volumes) to the eluted DNA. Mix, and centrifuge immediately at~ 15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.

T-cell hybridoma stimulation assay for DNA vaccines

This experiment was intended to determine if the T-cell receptor tolerated preferentially tolerated one of the linker sizes. The DNA vaccine first had to be transfected into the NOR-10 cell line before the cells could be used as targets in the stimulation assay. However, the T-cell hybridoma stimulation assay proved to be inappropriate for this task, thus all DNA vaccines were tested *in vivo*.

Day 1

___1. Aspirate NOR-10 growth media (NGM) (DMEM 20% FBS and 1% Penicillin-Streptomycin) from NOR-10 cells growing in a T75 flask

___2. Add 5 ml of Trypsin EDTA and incubate for 10 min at 37°C and 5% CO_2

___3. Add 20 ml of NGM to neutralize the trypsin

___4. Pellet cells at setting one in the clinical centrifuge for 10 min

___5. Resuspend the pellet in 10 ml NGM

___6. Count cells using the hemocytometer

___7. Seed 2.5 x 10^6 NOR-10 cells into a T25 flask and fill to 10 NOR-10 growth media

___8. Incubate overnight at 37°C and 5% CO_2

Day 2

___9. Dilute 12 μg of DNA (9 μg Vaccine and 3μg of peGFP-N2 (CloneTech)) with 500 μl of DMEM no serum; mix well

___10. Add 75 μl of SuperFect Transfection Reagent (Qiagen) and mix well

___11. Incubate at R1 for 5-10 min to allow complex formation

___12. While complex formation takes place, gently aspirate of media for the NOR-10 cells passed yesterday and wash with serum free DMEM or PBS

___13. Add 4.5 ml of DMEM 20% FBS (no antibiotics) to the DNA/SuperFect complex

___14. Transfer 5 ml of the DNA/Superfect media to the NOR-10 cells

___15. Incubate for 3 hrs at 37°C and 5% CO_2

___16. Aspirate off media and rinse 3 times with serum free DMEM or PBS

___17. Add 10 ml of NGM and incubate overnight

Day 3

___18. Aspirate media from NOR-10 cells growing in a T25 flask

___19. Add 2 ml of Trypsin EDTA and incubate for 10 min at 37°C and 5% CO_2

___20. Add 20 ml NGM to neutralize the trypsin

___21. Pellet cells at setting one in the clinical centrifuge for 10 min

___22. Resuspend the pellet in 10 ml NGM

___23. Count cells using the hemocytometer

___24. Seed 3 x 10^5 NOR-10 cells into a each well of a 12 well plate and fill to 2 ml with NGM.

___25. Incubate overnight at 37°C and 5% CO_2

Day 4

___26. Add Sendai Epitope to a final concentration of 2μM to the NOR-10 cells to be used as positive control.

___27. Incubate peptide NOR-10 cells for 2 hrs at 37°C and 5%CO_2

___28. In the mean time count T-cell hybridoma cells

___29. Dilute hybridoma cells to a final concentration of $1*10^6$ cells/ml

___30. Aspirate media off all NOR-10 cells including peptide pulsed cells

___31. Wash all NOR-10 cells twice with CTM (above)

___32. Add 2 ml of Tumor cells or Tumor media to the appropriate wells

___33. To the positive control add peptide to a final concentration of 2 μM

___34. Incubate cells at 37°C and 5%CO_2 for 18-24 hrs.

___35. Perform IL-2 ELISA (see PROTEIN VACCINE PROTOCOLS)

PROTOCOLS TAKEN FROM DR. WOODLAND'S LAB PROTOCOL BOOK AND PRESENTED IN A FORMAT SIMILAR TO THE OTHER PROTOCOLS IN THIS APPENDIX.

Lymphocyte processing and SEV9 K^b tetramer staining protocols

This protocol was used to assay if mice vaccinated with the β_2mL21SESP vaccine and the pUMVC3-mIL12 could mount an earlier immune response with a higher magnitude of CD8+ viral specific memory cells than mice vaccinated with the β_2mL21SESP and the pUMVC3-mIL12.

Collection of organs

___1. Mice are euthanized via the injection of 0.5 ml of Tribromoethanol via the intra-peritoneal route.

___2. When the mice are under the anesthetic effect of the Tribromoethanol they are cut in the auxiliary area with sharp scissors to nick the major auxiliary blood vessels. This allows for the bleeding out of the animal to minimize contamination of sampled organs by peripheral blood. The blood is allowed to drain from the animal for a minute or two.

___3. The mouse is then placed on its back with its head toward the technician and the skin gently removed over the neck and throat area. The trachea is then exposed by gently pulling back on the fat, muscle, and salivary glands located in the area from the top of the sternum to the lower portion of the mandible.

___4. The trachea is elevated using some curved forceps and a small cut is made on the upper surface of the trachea, taking care not to cut through the entire diameter of the trachea. This should provide a small hole in which the catheter for the collection of lymphocytes in the bronchiole and alveolar spaces can be inserted. The fluid removed in this manner is termed BAL for Bronchiole Alveolar Lavage.

___5. A 1cc syringe fitted with a plastic catheter and containing 1.0 ml of ice cold Hank's Balanced Salts Solution (HBSS) is gently threaded into the trachea toward the lungs. The 1.0 ml of HBSS is then injected into the lungs and then washed back and forth. A volume of approximately 0.75 to 0.9 ml should be recovered from the lung. This lavage of the lungs should be repeated 3 additional times to adequately remove all of the resident bronchiole and alveolar lymphocytes. Routinely, the BAL harvested from individual mice in a group is pooled for analysis. (Note: For the SEV9 K^b Tetramer Time Course Experiments, all mice were lavaged 4 times with 1.0 ml and samples from individual animals were pooled within groups.)

___6. The spleens of the mice are then removed by placing them in their right side and then carefully removing the skin on the left and making a small hole through the peritoneal wall above the spleen. The spleen is then exteriorized with forceps and cut free from associated blood vessels and connective tissues and placed in a tube containing 10 ml of HBSS on ice. Spleens from individual mice within a group are pooled for analysis.

___7. The Mediastinal Lymph Nodes (MLN) and Lungs are then harvested from the mice by placing them on their backs and removing the skin above the rib cage. A cut through the rib cage removing the sternum exposes the thoracic cavity. The ribs on the right side of the mouse are further trimmed to allow the better visualization of the MLN which can be found behind the right lung, medially and slightly anterior, along the back of the thoracic cavity. The MLN are grasped with forceps and gently cut to remove them and placed in 10 ml of cold HBSS. The MLN of individual mice are pooled within an experimental group. The Lungs are gently cut free and likewise pooled in 10 ml of cold HBSS.

Processing of tissues – single cell isolation, Gey's lysis & 40%-80% percoll of lung, & IgG primaria flask panning.

___8. The spleens, MLN's, and Lungs are gently pressed through a fine nylon mesh to achieve single cell suspensions. Briefly, the harvested organs are placed in 100 mm sterile petri dishes with the 10 ml of HBSS. With sterile forceps the organs are placed into nylon mesh bags, which are gently pressed with the plunger of a 5-10 ml syringe against the bottom of the petri dish. A single cell suspension should result in the surrounding HBSS, which can be harvested to a sterile centrifuge tube (Sp & MLN use 15 ml tube, Lung use 50 ml tube). The suspension from the lungs should be passed through a nylon mesh cells strainer to remove large pieces of tissue. The nylon mesh bags are rinsed with 5 ml of HBSS for Sp and MLN and 30 ml of HBSS for Lungs to recover as many lymphocytes as possible.

___9. The single cell suspensions (Sp, MLN, Lg, and BAL) are then centrifuged at 1200 rpm for 10 minutes at 4C (Program 2).

___10. The supernatant is then removed from the cell pellet via aspiration or gently tipping the tube. The cell pellets are gently flicked to resuspend and break up clumps.

___11. 2.0 ml of Gey's (below) solution is added to the Spleen and Lung cells, while 1.0 ml of Gey's is added to the MLN and BAL cells with gentle mixing. The cells are incubated at room temperature for 5 minutes and then topped off with HBSS to achieve a final volume of 15 ml per tube.

___12. The Gey's treated cell suspensions (Sp, MLN, Lg, and BAL) are then centrifuged at 1200 rpm for 10 minutes at 4C (Program 2).

___13. After centrifugation the supernatants are removed from the cell pellets as above and the Sp cells are resuspended in 10.0 ml of Complete Tumor Medium (CTM) and placed on ice. The MLN and BAL cells are resuspended with 1.0 ml of CTM and likewise placed on ice.

___14. The lung cells are resuspended with 1.0 ml of 80% Percoll per set of mouse lungs. Therefore, if 4 mice were harvested per pooled group, you need to suspend the lung cell pellet with 4.0 ml of 80% Percoll. The lung cell/80% Percoll solution is then added to 15 ml centrifuge tubes in 2.0 ml volumes.

___15. A 40% Percoll layer is then gently "floated" onto the surface of the lung cell/80% Percoll suspension. The best method to do this is to angle the 15 ml conical centrifuge tube to an angel of approximately 30 degrees and slowly pipette the 2.0 ml of 40% Percoll down the side of the tube. Once the 40% Percoll has been layered the tube can slowly be brought to the vertical position and you should see a sharp interface between the 40% and 80% layers. Be very gentle in handling the tubes so that you don't cause mixing to occur.

___16. The 40/80 Percoll gradients are then centrifuged for 25 minutes at 1650 rpm and 18C (Program 7 or 14)

___17. While the lung cells are spinning the Sp, MLN and BAL cells can be counted via the hemacytometer to determine cell numbers.

___18. The depletion of B-cells and Macrophages from the Spleen and MLN samples is accomplished via "panning" approximately 2.0E+07 cells onto T-75 Primaria flask which have been coated with anti-mouse IgG (Refer to W/B Protocol: B Cell Depletion by Panning: Goat Anti-Mouse IgG Coated Flasks). Briefly, a 10 ml suspension of $2.0x10^7$ cells in CTM is incubated in the coated Primaria flasks at 37°C, 10% CO_2, for one hour. The non-adherent cells are then gently

resuspended by rocking the flask and then washing with an additional 10 ml of CTM. The non-adherent cells are then centrifuged at 1200 rpm for 10 minutes at 4°C (Program 2). The cells are then resuspended in 1.0 ml of CTM and counted via the hemacytometer.

___19. After centrifugation of the lung cell Percoll gradients there should be a layer of lymphocytes at the interface of the 40% and 80% layers. These cells are gently removed using a Pasteur pipette and placing them into a 50 ml centrifuge tube.

___20. The Percoll enriched lymphocytes are washed with 25-30 ml of HBSS to remove the excess Percoll and centrifuged at 1200 rpm for 10 minutes at 4°C (Program 2). The supernatant is removed and the lung cells resuspended in 1.0 ml of CTM, placed on ice, and counted via the hemacytometer.

___21. When cell counts have been determined for all the tissues (Sp, MLN, Lg, and BAL), aliquots containing $5.0x10^5$ cells are added to replicate wells (1 well for each stain combination for each sample) of a U-bottom 96 well plate for NP/PA tetramer flow Cytometry staining. The maximum number of cells/well is $1x10^6$.

Tetramer (NP D^b & PA D^b) flow cytometry staining.

Staining Wash Buffer (SWB) (50 ml 10X PBS, 5 ml 10% Sodium Azide, 10 ml fetal calf serum, 435 ml ddH_2O)

___22. Add a volume of cells to achieve the appropriate number cells and wells in a 96-well round bottom plate (See Step #21).

___23. Spin the plate @ 1500 rpm for 3 minutes and 4°C (Program 3). Flick the plate.

___24. Resuspend the cell pellets in 50 µl of a 1:100 dilution of Fc Block (BD Pharmingen anti-mouse CD16/Cd32 Fc II/III) diluted in staining wash buffer (SWB).

___25. Incubate the plate on ice for 20 minutes.

___26. Add 150 µl of SWB to each well. Spin plate as above and flick.

___27. Add 100 µl / well of the appropriate dilution of SEV9 K^b tetramer (PE) stain which has been diluted in SWB. Incubate for 1 hour at room temperature in the dark.

___28. Add 100 µl / well of SWB to each well. Spin plate and flick.

___29. Add 200 µl / well of SWB to each well. Spin plate and flick.

___30. Add 100 µl / well of the appropriate dilution of secondary antibodies in SWB (FITC and TC/PC labeled). Incubate 20 minutes on ice in the dark.

___31. Add 100 µl / well staining wash buffer. Spin plate and flick.

___32. Wash 2 additional times with 200 µl /well of SWB.

___33. Resuspend the pellets in 125 µl staining wash buffer. The 125 µl will be transferred into microtubes prior to analysis.

___34. Run on FACS.

Viral titer

This protocol was used to assay if mice vaccinated with the β_2mL21SESP vaccine and the pUMVC3-mIL12 could clear virus quicker than mice vaccinated with the β_2mL21SESP and the pUMVC3-mIL12. Since you will be working with the Sendai virus all parts of this experiment except the initial 10 day egg incubation will need to be conducted in a bio-safety level 3 facility. Egg preparation

Fertile eggs can be stored between 50-60°F with ~ 72% relative humidity for up to 5 days before use; they must be turned daily.

___1. 10 days prior to use, place 216 eggs non-incubated but fertile (Charles River SpaFas, Inc.) into the incubator at 37.5°C with a relative humidity of 63%. (eggs can be used from day 9-11, but at no other time)

___2. In a dark room candle eggs. Put each egg against candle light and check for embryo status look for alive/dead and for live embryos mark the location of the air sack; discard dead eggs.

Chicken red blood cell preparation (maintain sterile conditions)

___3. Centrifuge chicken blood (Colorado Serum Co. CS 1131) at 1500 rpm for 5 minutes at 4°C. Remove plasma and white blood cell layer. Save RBC layer.

___4. Add 9ml of 0.85% saline for each ml of RBC and wash by centrifuging and discarding supernatant. Do this three times

___5. Pellet cells by centrifugation at 1500 rpm for 10 minutes at 4°C.

___6. You can store the blood for 5-7 days at this point.

___7. Create a 1% working solution before use.

Lung preparation

___8. Obtain fresh lung or frozen lung tissue

___9. Homogenize in 1 ml of antibiotic solution ($2x10^5$ units/ ml Penicillin G potassium (embryo tested) (Sigma P4687), 40mg/ml Streptomycin sulfate (Sigma S9137) and $2x10^4$ units/ml Polymyxin B sulfate (Sigma P4932)), using the tissue homogenizer

___10. Make 12 serial dilutions of each lung sample starting with undiluted

___11. Save any undiluted sample in case protocol needs to be repeated.

Egg injection

___12. Make a small hole with a hand Dremel tool in the egg at the location of the air sack.

___13. With the 25 gauge needle inject 100 µl of one of the sample dilutions.

___14. Seal the hole with melted paraffin.

Viral egg incubation

___15. Incubate eggs at 37°C for 48 hours.

___16. Transfer eggs to cold room for 24 hours

Haemagglutination assay

___17. Added 25 µl of PBS to each well, 2 wells per egg + 4 wells for positive control and 4 well for negative control that gives 440 well which is 4.6 plates.

___18. Draw out allantoic fluid with a glass Pasteur pipette through the small hole in the egg.

___19. Place 2 drops into each well for that sample. Use dilute Sendai virus for positive control and PBS for the negative control.

___20. Add 50 µl of the 1% chicken RBC solution.

___21. Incubate at RT for 30 minutes.

___22. Record results as positive if you see lattice formation (agglutination) or as negative if you see button formation (no agglutination)

The egg infectious dose (EID_{50}) is the highest dilution of virus that showed agglutination in 50% of the eggs.

Tribromoethanol 20 mg/ml

The protocol was used to prepare Tribromoethanol (Avertin), which was used to anesthetize and euthanize mice.

___1. In a graduated cylinder or glass bottle add 20 ml tert-amyl-OH (Fisher A730-1)

___2. Add 20 g 2,2,2-tribromoethanol (Aldrich T4840-2) to the alcohol.

___3. Swirl until the solid goes into solution at ambient temperature (this may take awhile).

___4. Slowly add 500 ml ddH_2O to flask on a hot plate while stirring (hot plate on low).

___5. Make sure that salts stay in solution before you add more H_2O.

___6. Bring water to 1 L.

___7. Turn the hot plate off when all of the solid has gone into solution.

___8. Stir O/N.

___9. Check that the 2,2,2-tribromoethanol has not precipitated out of solution. If necessary, turn the hot plate back on until the solid has gone back into solution.

___10. Bottle-top filter (0.22 μm) to remove eluate.

___11. Aliquot into glass bottles and label the bottles with the date made and your initials.

___12. Store the Avertin in the refrigerator in the bottom drawer to minimize the amount of light contacting the solution.

___13. For each batch of Tribromoethanol made, test 5 C57Bl/6 mice with 0.20cc, 0.25cc, 0.30cc of solution (also called Avertin). Note how lightly the mice are asleep by squeezing the hind feet. Also note if any of the mice do not wake up.

Lysis of red blood cells

This protocol was used to lyse red blood cells in collected tissues.

___1. Allow an appropriate aliquot of Gey's Solution (below) to warm to room temperature from 4°C and, if necessary, equilibrate the pH to ~7.0 by incubation in a 10% CO_2 incubator.

___2. Prepare a single cell suspension of spleen cells and spin down 400g for 10 min.

___3. Dry resuspend the pellet and add Gey's Solution (1-2 ml/spleen).

___4. Incubate 5 minutes at room temperature and add 20 ml of HBSS to wash the cells.

Gey's solution (buffered ammonium chloride)

This protocol was used to prepare Gey's solution, which was used to lyse red blood cells.

___1. Combine 8.0 g NH_4Cl (Fisher 42328-0010), 0.5 g $KHCO_3$ (Sigma P-9144), 0.5 ml 0.5% Phenol Red (Sigma P-0290)

___2. Added ddH_2O to a final volume of 500 ml

___3. Sterile filter and store at 4^oC.

Appendix II: Primers

Primer Name: _21INSERT5_____

Manufacturer: _SIGMA_____

Who ordered it: _Danny Piper_____

Length (nt): ___69_____

Molecular Weight: ___21544.4_____

Melting Temp (ºC): ___90.4_____

Concentration: _ _stock 100 μM, working 10 μM_ ____

Sequence:

5'CCGGT TTATA TGCTT TTGCG CCAGG AAATT ATCCC GCTTT AGGAG GTGGT GGAGG TAGTG GTGGA GGAG 3'

Comments/Use:

21INSERT5 was ordered along with 21insert3 to allow one to increase the pVAX1 β_2mL15SESP to a 21 AA linker. There are AGE1 and BAMH I overhangs when the two anneal together

Primer Name: 21Insert3

Manufacturer: Sigma

Who ordered it: Danny

Length (nt): 69

Molecular Weight: 20966.3

Melting Temp (ºC): 89.3

Concentration: Stock 100 µM, Working 10 µM

Sequence:

5’ GATCC TCCTC CACCA CTACC TCCAC CACCT CCTAA AGCGG GATAA TTTCC TGGCG CAAAA GCATA TAAA 3’

Comments/Use:

21INSERT3 was ordered along with 21insert5 to allow one to increase the pVAX1 β_2mL15SESP to a 21 AA linker. There are AGE 1 and BAMH I overhangs when the two anneal together.

Primer Name: ForPVAX

Manufacturer: Sigma Genosys

Who ordered it: Danny Piper

Length (nt): 18

Molecular Weight: 5493.6

Melting Temp (ºC): 62.1

Concentration: Stock 150 μM, working 15 μM

Sequence:

5’ AGACC CAAGC TGGCT AGC 3’

Comments/Uses:

Forward primer for sequencing pVAX1 vector constructs

Primer Name: RevPVAX

Manufacturer: Sigma Genosys

Who ordered it: Danny Piper

Length (nt): 18

Molecular Weight: 5564.6

Melting Temp (ºC): 64.5

Concentration: Stock 150 µM, working 15 µM

Sequence:

5’ AGTCG AGGCT GATCA GCG 3’

Comments/Uses:

Reverse primer for sequencing pVAX1 vector constructs.

Primer Name: ForPCT7

Manufacturer: Sigma Genosys

Who ordered it: Danny Piper

Length (nt): 18

Molecular Weight: 5589

Melting Temp (ºC): 59.8

Concentration: Stock 150 μM, working 15 μM

Sequence:

5’ AGCAT GACTG GTGGA GTGGA CAG 3’

Comments/Uses:

Forward primer for sequencing pCRT7/NT vector constructs.

Primer Name: EKDEP

Manufacturer: Sigma

Who ordered it: Jamie Caras

Length (nt): 48

Molecular Weight: 24901

Melting Temp (ºC): 99.8

Concentration: Stock 150 μM, working 15 μM

Sequence:

5'– AGCAG CGAGC TCATC GACGA CGACG ACAAG TTCGC TCCGG GTAAC TACCC GGCTC TGGGA GGTGG CGGAT CCGGT GGC –3'

Comments/Uses

Second primer in Protein PCR scheme, anneals to the linker and adds the epitope and enterokinase site. Has a high melting temperature and may form dimers.

Primer Name: HISTAG

Manufacturer: Sigma Genosys

Who ordered it: Jamie Caras

Length (nt): 65

Molecular Weight: 18863

Melting Temp(ºC): 94.5

Concentration: Stock 150 μM, working 15 μM

Sequence:

5'– ATCTG GCCCA GCCGG CCCAG CATAT GGGCC ATCAT CATCA TCATC ATAGC AGCGA GCTCA TCGAC –3'

Comments/Uses:

Adheres to EKDEP and adds a Histag and Nde I endonuclease site

Primer Name: T7PRIMER

Manufacturer: Sigma Genosys

Who ordered it: Danny Piper

Length (nt): 22

Molecular Weight: 6743

Melting Temp(ºC): 56.0

Concentration: Stock 150 μM, working 15 μM

Sequence:

5’ GTAAT ACGAC TCACT ATAGG GC 3’

Comments/Uses:

Standard primer for many vectors and the down stream primer in the PCR reactions

Primer Name: β2MLINK

Manufacturer: Sigma Genosys

Who ordered it: Jamie

Length (nt): 84

Molecular Weight: 26292

Melting Temp (ºC): 101.7

Concentration: Stock 150 μM, working 15 μM

Sequence:

5'– GGAGG TGGCG GATCC GGTGG CGGAG GAAGC GGCGG AGGTG GCAGC GGACG TGGCG GAAGC GGAAT CCAGA AAACC CCTCA AATT –3'

Comments/Uses:

Binds to the 5′ prime end of β_2m and adds the sequence that codes for a 21AA linker

Primer Name: β2MLINK15

Manufacturer: Sigma Genosys

Who ordered it: Ralphie

Length (nt): 66

Molecular Weight: 20552

Melting Temp (ºC): 98.5

Concentration: Stock 150 μM, working 15 μM

Sequence:

5'-- GGAGG TGGCG GATCC GGTGG CGGAG GAAGC GGCGG AGGTG GCAGC ATCCA GAAAA CCCCT CAAAT T --3'

Comments/Uses:

Binds to β_2m and adds a 15 AA linker.

Primer Name: Link10

Manufacturer: Sigma Genosys

Who ordered it: Ralphie

Length (nt): 48

Molecular Weight: 14880

Melting Temp (ºC): 94

Concentration: Stock 150 µM, working 15 µM

Sequence:

5` -- GGAGG TGGCG GATCC GGTGG CGGAA GCGGA ATCCA GAAAA CCCCT CAA --3`

Comments/Uses:

Binds to β_2m and adds the code for a 10 AA linker to the 5′ end.

Primer Name: DNAVACCINESANDIMEPITOPESIGNALPEPTIDE

Manufacturer: Sigma Genosys

Who ordered it: Danny Piper

Length (nt): 73

Molecular Weight: 22565

Melting Temp (ºC): 85.8

Concentration: Stock 150 µM, working 15 µM

Sequence:

5` -- TTTAG TAAGT TTAAC CGGTT TATAT GCTTT TGCTC CTGGT AATTA TCCAG CTTTA GGAGG TGGCG GATCC GGT --3`

Comments/Uses:

Adhere to the 5` end of any of the linkers and add the Sendai epitope and part of the signal epitope. May form dimers.

Primer Name: DNAVACCINESIGNALPEPTIDEPVAX

Manufacturer: Sigma Genosys

Who ordered it: Danny Piper

Length (nt): 75

Molecular Weight: 23137

Melting Temp (ºC): 77.4

Concentration: Stock 150 µM, working 15 µM

Sequence:

5` -- AATAT AATAA CTTAA GCTAG CTAAC ATGGC GAGAT CAGTA ACTCT TGTAT TTCTA GTTTT AGTAA GTTTA ACCGG -- 3`

Comments/Uses:

Attaches to the part of signal peptide and add the remaining part of signal peptide and necessary Nhe I endonuclease site to ligate into pVAX1 vector

Primer Name: CONTROL3

Manufacturer: SIGMA

Who ordered it: Larisa Watson

Length (nt): 69

Molecular Weight: 20984.3

Melting Temp (ºC): 88.0

Concentration: stock 100 µM, working 10 µM

Sequence:

5'GATCCTCCTCCACCACTACCTCCACCACCTCCTAAAGCAAGGGCTCGTATA
GACGCAAAAGCATATAAA 3'

Comments/Use:

CONTROL3 was ordered along with CONTROL5 to allow one to test the specificity of the T cell hybridoma for the Sendai epitope in the DNA vaccines. The epitope will be used as a control. The epitope expressed will be FASIRALAL, in place of the Sendai epitope. There are AGE1 and BAMH I overhangs when the two anneal together.

Primer Name: CONTROL5

Manufacturer: SIGMA

Who ordered it: Larisa Watson

Length (nt): 69

Molecular Weight: 21526.3

Melting Temp (ºC): 89.1

Concentration: Stock 100 µM, Working 10 µM

Sequence:

5'CCGGTTTATATGCTTTTGCGTCTATACGAGCCCTTGCTTTAGGAGGTGGTGG
AGGTAGTGGTGGAGGAG 3'

Comments/Use:

CONTROL5 was ordered along with CONTROL3 to allow one to test the specificity of the T cell hybridoma for the Sendai epitope in the DNA vaccines. The epitope will be used as a control. The epitope expressed will be FASIRALAL, in place of the Sendai epitope. There are AGE1 and BAMH I overhangs when the two anneal together.

Primer Name: NO Epitope3

Manufacturer: SIGMA

Who ordered it: Larisa Watson

Length (nt): 42

Molecular Weight: 12572

Melting Temp (ºC): 82.1

Concentration: Stock 100 µM, Working 10 µM

Sequence:

5'GATCCTCCTCCACCACTACCTCCACCACCTCCAGCATATAAA 3'

Comments/Use:

No Epitope 3 was ordered along with No Epitope 5 to allow one to test if the linker alone could elicit a Sendai specific immune response in the DNA vaccines. The no epitope will be used as a negative control in T-cell stimulation experiments. There are AGE1 and BAMH I overhangs when the two anneal together.

Primer Name: NO Epitope5

Manufacturer: SIGMA

Who ordered it: Larisa Watson

Length (nt): 42

Molecular Weight: 13257.2

Melting Temp (ºC): 84.1

Concentration: Stock 100 µM, Working 10 µM

Sequence:

5'CCGGTTTATATGCTGGAGGTGGTGGAGGTAGTGGTGGAGGAG 3'

Comments/Use:

No Epitope 5 was ordered along with No Epitope 3 to allow one to test if the linker alone could elicit a Sendai specific immune response in the DNA vaccine. The no epitope will be used as a negative control in T-cell stimulation experiments. There are AGE1 and BAMH I overhangs when the two anneal together.

Primer Name: _ForLARISA_____

Manufacturer: _SIGMA_____

Who ordered it: _Larisa Watson_____

Length (nt): ___56_____

Molecular Weight: ___17310.8_____

Melting Temp (ºC): ___89.4_____

Concentration: ___100 μM_____

Sequence:

5'CATTGACGACGACGATAAGTTCGCATCTATACGCGCCCTTGCTTTAGGAGG TGGGG 3'

Comments/Use:

ForLARISA was ordered along with RevLARISA and when combined will form Sal1 and BAMH I overhangs. The annealed product can be put into the pCRT7/NT β2mL15SEHT cut with Sal I and BAMH I to replace the Sendai Epitope with a dummy epitope with a linker size of 21 AA. The dummy epitope will be used as a negative control in T-cell stimulation experiments. The procedure did not work.

Primer Name: RevLARISA

Manufacturer: SIGMA

Who ordered it: Larisa Watson

Length (nt): 64

Molecular Weight: 19640.3

Melting Temp (ºC): 90.8

Concentration: 100 μM

Sequence:

5'GATCCCCCAC CTCCTAAAGC AAGGGCGCGT ATAGATGCGA ACTTATCGTC GTCGTCAATG AGCT 3'

Comments/Use:

RevLARISA was ordered along with ForLARISA and when combined will form Sal1 and BAMH I overhangs. The annealed product can be put into the pCRT7/NT β2mL15SEHT cut with Sal I and BAMH I to replace the Sendai Epitope with a dummy epitope with a linker size of 15 AA. The dummy epitope will be used as a negative control in T-cell stimulation experiments. This procedure did not work.

Primer Name: PCRLAR15

Manufacturer: SIGMA

Who ordered it: Danny Piper

Length (nt): 61

Molecular Weight: 19640.3

Melting Temp (ºC): 90.8

Concentration: 100 μM

Sequence:

5'ATTGACGACGACGATAAGTTCGCATCTATACGTGCCCTTGCTCTGGGAGGT GGCGGATCCG 3'

Comments/Use:

PCRLAR 15 encodes for the Enterokinase site and the Dummy epitope FASIRALAL. During a one step PCR with pCRT7/NT β2mL15SEHT as a template and the T7 primer as the down stream primer, it one can exchange the Sendai epitope with the dummy epitope. The dummy epitope will be used as a negative control and has a linker size of 15 AA.

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Vita

John Daniel Piper (Danny) was born on October 9, 1970 in Hinsdale, IL to his loving parents, Jack and Sandra Piper, and older brother Andy. One year later his brother, Ted, joined the family. In 1976, the Piper family moved to Westchester, PA and then again in 1978 the family moved to Sandra's hometown of Roswell, GA. All three Piper boys attended St. Pius X Catholic High School in Atlanta, GA. Just prior to graduating from high school, in April of 1989, a baby girl, Holly, became Danny's third sibling and only sister. Also that year Danny joined his older brother at the Georgia Institute of Technology (GA Tech). While at GA Tech, Danny participated on the Rowing Team and spent four years on a quest for a gold medal at the Dad Vail National Collegiate Regatta, to walk away with a only a silver medal but with the friendships of a lifetime. Also, while at GA Tech, Danny joined Dr. Pat Gillis's lab to explore the use of high energy gases to etch silicon wafers and published a paper on the topic (Gillis, Choutov et al. 1995). In 1996, Danny graduated from GA Tech with two bachelor degrees, a B CHE and a BS CHEM. He also obtained a high school teaching certificate after completing courses at Agnes Scott College, Decatur, GA. In 1997, he started graduate school in the Department of Pharmacology at the University of Texas. In 1998, he switched his field to Biochemistry and joined Dr. G. Barrie Kitto's lab. From 1999 until 2002 he was supported by a NIH Biotechnology Training Grant. While in graduate school he completed a summer internship with AtheroGenics, where he explored the regulation of TNF-α induced expression of VCAM-1. In September 2001, he began dating Jennifer Ann Donnelly, a most beautiful young lady with the intellect and

personality to match. On January 11, 2002, Danny put a ring on her finger and they were married on August 10th of that same year. Upon graduation Danny and Jenni will be moving back to Roswell, GA and Danny will be returning to work at AtheroGenics.

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